

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/18, A61L 27/00, C12N 5/08	A1	(11) International Publication Number: WO 96/30038 (43) International Publication Date: 3 October 1996 (03.10.96)
(21) International Application Number: PCT/US96/04326 (22) International Filing Date: 29 March 1996 (29.03.96) (30) Priority Data: 08/413,047 29 March 1995 (29.03.95) US 08/622,860 29 March 1996 (29.03.96) US (71) Applicant: THE ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021-6399 (US). (72) Inventors: HEMMATI-BRIVANLOU, Ali; Apartment 6D, 325 East 84th Street, New York, NY 10028 (US). WILSON, Paul; Apartment 10A, 200 East 70th Street, New York, NY 10021 (US). (74) Agents: DAVIS, Michael, D. et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).		(81) Designated States: AU, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: PEPTIDE GROWTH FACTOR HAVING EPIDERMAL INDUCING ACTIVITY (57) Abstract <p>The invention relates to a method for suppressing autoneuralization and inducing epidermis using a peptide growth factor which is a gene product of a member of the TGF-β gene family. The method is useful in wound healing, skin culture technology, and treatment of tumors derived from neural tissue.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

PEPTIDE GROWTH FACTOR HAVING EPIDERMAL INDUCING ACTIVITY

BACKGROUND OF THE INVENTION

5 1. Field of the Invention

This invention relates generally to gene products of the TGF- β gene family, to methods and compositions utilizing such factors, and to the antibodies reactive toward them, in assays and for diagnosing, preventing and/or treating cellular debilitation, derangement or dysfunction.

10

2. Description of the Related Art

The induction of the nervous system, one of the earliest and most dramatic events of vertebrate development, has challenged and frustrated embryologists since the organizer graft experiments of Spemann and Mangold. Classical work established
15 that the gastrula stage ectoderm of amphibian and other vertebrate embryos gives rise to the neural plate in response to signals from the adjacent dorsal mesoderm (Spemann's organizer). In the absence of this influence, as on the ventral side or in explants made before gastrulation, the ectoderm differentiates only as epidermis. Thus, development as epidermis was generally assumed to be a fall-back, or
20 default fate for the gastrula ectoderm requiring no cell-cell communication, while neural specification was contingent on receipt of signals. However, much effort over several decades failed to identify the chemical substances responsible for neural induction in the embryo, although a variety of rather curious materials were found to be able to neuralize salamander ectoderm. Recent studies of the
25 amphibian embryo have identified three diffusible factors with neural inducing ability: noggin (Lamb *et al.*, 1993), follistatin (Hemmati-Brivanlou *et al.*, 1994) and chordin (short gastrulation) (Sasai *et al.*, 1995; Sasai *et al.*, 1994). All three factors mimic the signal(s) which emanates from the organizer and converts ventral ectoderm (epidermis) to dorsal ectoderm (nervous tissue).

30

Other recent work has led to a second promising molecular candidate for a neural inducing signal and at the same time suggested a new twist on the long-held classical model of neural and epidermal specification. First, Grunz and others

revealed that *Xenopus* ectoderm cultured during early gastrula stages as a dispersed cell population formed neural tissue even though it receives no signals from the mesoderm during this period. More recently, Brivanlou and Melton discovered that injection of a dominant-negative form of the activin receptor could neuralize
5 ectodermal explants, again in the apparent absence of mesoderm. Finally, the activin antagonist follistatin could also cause neural differentiation. These findings led Brivanlou and Melton to propose that the cells of the early gastrula animal cap are disposed to form neural tissue, in the absence of further influences. In this sense one could speak of a default neural fate for the ectoderm. Epidermal
10 specification, and thus the inhibition of neural fate, results from cell-cell communication within the prospective ectoderm. When this signalling is interrupted, by dispersing the cells or by molecular antagonists, neural tissue forms. Neural induction by the dorsal mesoderm, in this model, would work in the same way, that is by blocking epidermalizing signalling within the animal cap.
15 Since both the truncated activin receptor and follistatin could accomplish this, activin seemed likely to be the factor that mediated epidermal specification. The further discovery that follistatin was expressed in the organizer region in *Xenopus*, from where it could act to block activin signaling in the dorsal ectoderm and thus permit neural tissue to form, naturally suggested follistatin as an endogenous
20 neural inducer. Although these were enticing speculations, there was no direct evidence that activin could act to specify epidermis, or that such an inducer existed at all.

The BMPs are a set of closely related proteins that form a subgroup within the
25 larger TGF- β superfamily of secreted growth factors that also includes activin and the TGF's proper. First purified from bone as activities capable of promoting bone regrowth, the BMPs have more recently been found in early vertebrate embryos, where they appear to play a variety of roles. In *Xenopus*, several groups have shown that BMPs 2 and 4 are capable of inducing ventral mesoderm, as well
30 as ventralizing mesoderm induced by activin.

Recent work showing that BMP-4 is expressed in the ventral marginal zone, and that a dominant negative version of a BMP receptor has strong dorsalizing effects on early embryos lends further support to the idea that BMP-4 acts in vivo to ventralize the marginal zone.

5

There is accumulating evidence that genes belonging to the Transforming Growth Factor- β (TGF- β) family are important regulators of many morphogenetic events during early vertebrate embryogenesis (for review see Whitman and Melton, 1989). For example, TGF- β 1 in combination with basic fibroblast growth factor (Kimelman and Kirschner, 1987), TGF- β 2 alone (Rosa et al., 1988) and activin A (XTC-MIF) (Asashima et al., 1990); Smith et al., 1990; van den Eijnden-Van Raaij et al., 1990) can induce mesodermal differentiation and specific gene expression in isolated *Xenopus* animal caps which would otherwise form ectoderm. Furthermore, studies localizing TGF- β 1, - β 2, and - β 3 transcripts and/or protein have revealed temporal and spatial patterns of expression consistent with many roles for TGF- β -related genes in murine embryogenesis (Heine et al., 1987; Lehnert and Akhurst, 1988; Pelton et al., 1989, 1990). A group of the TGF- β gene family, whose members show greatest homology to the *Drosophila* *Decapentaplegic* (*dpp*) (Padgett et al., 1987) and *Xenopus* *Vg-1* (Weeks and Melton, 1988) genes includes the *Bone Morphogenetic Proteins* (BMP) 2,3 (osteogenin), and 2b (now known as BMP-4) (Wozney et al., 1988; Luyten et al., 1989), as well as murine *Vgr-1* (Lyons et al., 1989a), *Osteogenic Protein 1* (Ozkaynak et al., 1990), and *GDF-1* (Lee, 1990). Recent studies have localized *Vgr-1* RNA to mouse oocytes, suprabasal layers of keratinized epithelium and hypertrophic cartilage in developing bone (Lyons et al., 1989b). A different pattern of expression is seen for BMP-2, which is localized to the apical ectodermal ridge of limb buds, development hair and whisker follicles, condensing, precartilaginous mesenchyme and the myogenic layer of the atrioventricular cushions of the developing heart, as well as other areas undergoing morphogenesis during murine development (Lyons et al., 1989b, 1990).

The original BMP-4 CDNA (formerly called BMP-2b) was isolated because of its homology to BMP-2 (W zney et al., 1988). This cDNA encodes a protein whose carboxy terminus (TGF- β conserved region) shows 92% amino acid identity to the corresponding region of BMP-2. The carboxy termini of BMP-2 and BMP-4 are
5 more closely related to the corresponding region of *Drosophila* dpp protein than to any other known member of the TGF- β gene family.

Like the other TGF- β -related genes, BMP-4 transcripts are localized to specific regions of the developing mouse that are undergoing morphogenesis. These
10 include cells of newly formed mesoderm in the posterior primitive streak region of 8.5 days, *p.c.* embryos, as well as cell types involved in craniofacial development, limb bud formation, cardiac development and neuroepithelium associated with pituitary development. In some of these tissues, it appears that the distribution of BMP-4 transcripts overlaps that of BMP-2. In other cases, the
15 expression patterns of the two genes are different. In the central nervous system (CNS), the pattern of BMP-4 expression is also distinct from that of Vgr-1. Vgr-1 transcripts have been localized in the roof plate and in neuroepithelium adjacent to the floor plate within the developing CNS.

20 BMP-4 is expressed both in the AER at 10.5 days *p.c.* and in the mesenchyme, apparently in an anterior-posterior and distal-proximal gradient (Fig. 7). The possibility therefore exists that BMP-2 and BMP-4 cooperate to influence early limb development. Moreover, heterodimers of BMP-2 and BMP-4 could be formed within the AER. TGF- β -related growth factors have been shown to have
25 clearly different activities when acting as a homodimer or heterodimer. For example, inhibin β homodimers selectively stimulate FSH release in the pituitary gland, while inhibin β/α heterodimers inhibit such secretion (Hsueh et al., 1987). BMP-4 could therefore have differing effects if it is a homodimer in the mesenchyme or complexed with BMP-2 in the AER.

30

The fact that BMP-4 is apparently expressed in a gradient in the mesenchyme invites comparison with the gradients detected for homeobox-containing genes such as members of the Hox 5 cluster (Dollé et al., 1989a) and X1hbox 1/Hox 3.3 (Oliver et al., 1988). These results raise the possibility that BMP-4 is part of a cascade of polypeptide signalling molecules, homeobox and retinoic acid receptor (Dollé et al., 1989b) genes involved in establishing pattern within the developing limb. Support for complex networks of interaction between such molecules comes from recent observations that the homeobox-containing gene, *Ubx*, activates *dpp* expression, with *dpp* in turn regulation *wg* and *lab* in the *Drosophila* midgut (Immergluck et al, 1990) and that XTC-MIF (activin A) induces a characteristic level of *Xhox 3* expression in *Xenopus* animal caps (Ruiz i Altab and Melton, 1989).

BMP-4 expression in the mesenchyme is transient, since no hybridization signal is found in the mesenchyme of stage 2 follicles. From this localization pattern, BMP-4 could be acting as a signalling molecule, instructing the overlying ectoderm. Consistent with this view, mesenchymal condensations can induce hair follicle formation when transplanted under previously hairless epithelium (Kollar, 1980). The expression of BMP-4 in facial regions of the early mouse embryo suggests a role of the gene in craniofacial morphogenesis. Expression in the early otic vesicle also raises the possibility that BMP-4 is involved in development of the inner ear.

BMP-4 expression is often coordinated in a temporal and spatial manner with expression of other members of the TGF- β gene family, particularly the closely related gene, BMP-2. While the full significance of these patterns must await studies with the purified proteins and localization of the cellular receptors for each molecule, they nevertheless strongly suggest that members of the TGF- β family function in inductive tissues interact during the establishment of several specialized organ systems within the developing embryo.

There are at least seven BMP proteins which have been elucidated by molecular cloning. BMPs 2-7 form a unique subfamily within the TGF- β gene family (Wozney (1993) "Bone Morphogenetic Proteins and Their Gene Expression," in *Cellular and Molecular Biology of Bone*, M. Noda, ed., Academic Press, Inc., New York). The BMPs act as differentiation factors, causing induction and increased expression of multiple differentiated phenotypes in mesenchymal cells. The BMPs are also involved in a variety of developmental processes including (1) the formation of the skeleton; (2) the development of organ and tissue systems that form via mesenchymal-epithelial interactions; and (3) and possibly the delivery or interpretation of positional information (Wozney, 1993).

The sequences of BMP-2 and BMP-4 are disclosed in U.S. Patent Nos. 5,013,649 and 5,166,058, respectively, which are hereby incorporated by reference in their entirety. A comparison of the amino acid sequences of the human BMPs indicates that significant amino acid identity exists among all the BMPs in the carboxy terminal region of the proteins. This region contains seven cysteine residues, whose presence and relative positions are conserved among all reported members of the TGF- β superfamily. BMP-2 and BMP-4 are 92% identical in the seven-cysteine region. All of BMPs 2-7 contain hydrophobic secretory leader sequences and substantial propeptide regions. The mature portion of each molecule resides in the carboxy terminus of the prepropeptide and includes the seven-cysteine domain. The amino-terminal domains of the mature BMPs are basic, processing occurring at the consensus sequence of Arg-X-X-Arg. The BMPs are glycosylated, BMP-4 having two N-linked glycosylation sites. BMP-4 is localized to chromosome 14, and may be a candidate for the *pugnose* locus, which results in abnormalities in skull bone development (Wozney, 1993).

Each mature, active BMP is a dimeric molecule containing two of these polypeptides. Potential also exists for formation of heterodimeric forms of the BMPs, which may possess different specific activities, receptor binding characteristics, or entirely novel activities relative to the homodimeric forms

(Wozney, 1993). BMP-2, BMP-4, BMP-5, BMP-6 and BMP-7 all induce some alkaline phosphatase expression in W-20-17 cells but vary in specific activity. The difference in activity between the less active BMP-2 and more active BMP-4 can be obviated by removal of their amino-terminal domains with trypsin, suggesting a
5 role for this domain in receptor binding or ligand presentation. There is a report of the presence of high-affinity binding sites for BMP-4 on MC3T3-E1 and NIH3T3 cells. By cross-linking experiments, binding proteins of 200 and 70 Kd are present on MC3T3-E1 cells, whereas proteins of 200 and 90 Kd are present on NIH3T3 cells (Wozney, 1993).

10

Specific embryonic tissue with "organizing" activity that exists in amniotes include Hensen's node in birds and the node in mammals that can give rise to a secondary axis when transplanted to an ectopic site of host embryos (Beddington, 1994; Waddington and Schmidt, 1933). Hensen's node from chick embryos can induce
15 neural tissue when recombined with *Xenopus* ectoderm (Kintner and Dodd, 1991). These similarities suggest that factors involved in these types of inductive interactions may be conserved among different vertebrate species.

TGF- β family members have been implicated in the differentiation processes of
20 P19 cells (reviewed in Mummery and van den Eijnden-van Raaij, 1993). There is evidence that activin promotes proliferation of undifferentiated P19 cells (Hashimoto *et al.*, 1990; Schubert *et al.*, 1990). Activin has also been shown to inhibit neural differentiation induced by RA (Hashimoto *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1991), implicating activin as a neural inhibitor in
25 mammalian cells. This inhibitory role of activin is consistent with studies of a neuroblastoma cell line, IMR-32, in which follistatin stimulates neural differentiation (Hashimoto *et al.*, 1992). Undifferentiated P19 cells express both activin b_6 and follistatin RNA (van den Eijnden-van Raaij *et al.*, 1992). The levels of activin b_6 are reduced in response to RA treatment while follistatin
30 mRNA expression is enhanced within 24 hours of RA treatment but disappears after 2 days (Hashimoto *et al.*, 1992). These observations suggest that the early

stages of neural differentiation in P19 cells may require the functional elimination of TGF- β s, including activins. P19 cells also express both the type I and the type II activin receptors (Kondo *et al.*, 1989). Collectively, these data support the conclusion that TGF- β signaling in P19 cells is intact and suggest a role of TGF- β signaling in the neural differentiation of mammalian cells.

Neural inducers have long been sought by exposing explanted ectoderm to candidate substances or extracts. The hypothesis that it is epidermal rather than neural specification which requires cell-cell communication, in this case local signalling among ectodermal cells, leads to a different assay that would not have been suggested by the traditional model. Since ectoderm dispersed for several hours adopts a neural fate (see above), it should be possible to add factors back to such a culture system in an effort to restore the epidermal specification of the intact cap. Identification of an epidermal inducer in this way would lend strong support to the default neural model, by showing that epidermis can in fact be an induced tissue. Moreover, such a finding could guide work on neural induction, focussing attention on antagonists of the epidermal inducer.

Although BMP-4 has been reported to mediate cell death in the developing hindbrain later in development, no role for BMPs in the earliest development of the ectoderm had been suggested until the present invention.

SUMMARY OF THE INVENTION

Accordingly, a major object of the present invention is to provide a method of preventing and/or treating cellular debilitations, derangements and/or dysfunctions and/or other disease states in mammals, including administering to a mammal a therapeutically effective amount of a material selected from the group consisting of a gene product of a member of a TGF- β gene family or active fragment thereof, an agent capable of promoting the production and/or activity of the gene product of a member of the TGF- β gene family, an agent capable of mimicking the

activity of the gene product of a member of the TGF- β gene family, an agent capable of inhibiting the production of the gene product of a member of the TGF- β gene family, and mixtures thereof, or a specific binding partner thereto, wherein the gene product of a member of the TGF- β gene family induces formation of epidermis.

Another object of the invention is to provide a method for producing artificial skin, including adding an amount of a gene product of a member of a TGF- β gene family or active fragment thereof sufficient to form epidermis to a culture of cells which are induced to form epidermis.

Still another object of the invention is to provide a method of inhibiting neural induction, including administering to a mammal an amount sufficient to inhibit neural induction of a material selected from the group consisting of a gene product of a member of a TGF- β gene family or active fragment thereof, an agent capable of promoting the production and/or activity of the gene product of a member of the TGF- β gene family, an agent capable of mimicking the activity of the gene product of a member of the TGF- β gene family, an agent capable of inhibiting the production of the gene product of a member of the TGF- β gene family, and mixtures thereof, or a specific binding partner thereto.

Yet another object of the invention is to provide a method of inducing epidermis formation, including administering to a mammal an amount sufficient to induce epidermis formation of a material selected from the group consisting of a gene product of a member of a TGF- β gene family or active fragment thereof, an agent capable of promoting the production and/or activity of the gene product of a member of the TGF- β gene family, an agent capable of mimicking the activity of the gene product of a member of the TGF- β gene family, an agent capable of inhibiting the production of the gene product of a member of the TGF- β gene family, and mixtures thereof, or a specific binding partner thereto.

A further object of the invention is to provide a method for identifying a compound which is an antagonist of a gene product of a member of a TGF- β gene family or active fragment thereof for inducing epidermis including the steps of:

- 5 A. contacting a biological sample containing cells to be induced with the gene product of a member of a TGF- β gene family and a candidate compound; and
- B. detecting whether the level of epidermis induction by the gene product of a member of a TGF- β gene family and a candidate is decreased relative to the level of epidermis induction in a control biological sample;
- 10 wherein a decrease in the level of epidermis induction by the gene product of a member of a TGF- β gene family indicates that the candidate is an antagonist of the epidermis inducing activity of the gene product of a member of a TGF- β gene family.

- 15 Another object of the invention is to provide a method for inducing expression of an epidermal cell surface marker in epidermal progenitor cells, including contacting said cells with an amount of a gene product of a TGF- β gene family or active fragment thereof sufficient to induce expression of the cell surface marker.

- 20 Briefly, the present invention features a gene product of a member of the TGF- β gene family, which can be used to induce the formation of epidermis, and suppress autoneuralization of progenitor cells. In particular, the invention relates to bone morphogenesis protein-4 (BMP-4), and its use in treating diseases or debilitations relating to the skin and nervous system. BMP-4, but not activin, restores
- 25 epidermal differentiation to dispersed gastrula ectoderm. BMP-4 can also be used to produce artificial skin for the testing of drugs, cosmetics and other medicaments, and to induce or suppress the expression of certain cell surface molecules which are associated with skin and neural tissue, respectively. In addition, the invention relates to antagonists of BMP-4, which can act to reverse
- 30 or diminish the action of BMP-4 under certain circumstances.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1. (A) Prolonged dispersal neuralizes late blastula ectoderm. Late stage 9 animal caps were disaggregated in calcium and magnesium-free media. Cells were reagggregated immediately (0 hours), or after 1-5 hours of dispersed culture. Reagggregates were cultured to the end of neurulation, when RNA was extracted for RT-PCR. Ectoderm subjected to dispersal and immediate reagggregation differentiates as epidermis, as do intact animal caps, expressing the epidermis-specific cytokeratin. The general neural marker NCAM is not present. Dispersal for 2 hours or less has no effect. However, after 3 hours of dispersed culture, ectoderm is neuralized, and reagggregates subsequently express NCAM, and not epidermal keratin. Dispersed cells were cultured in Ca and Mg-free MBSH; otherwise disaggregation and reagggregation were performed as in ref. RNA extraction and RT-PCR were also as in ref -, with the single modification that random hexamers rather than oligo dT were used to prime reverse transcription. (B) Autoneuralization by prolonged dispersion does not require secondary cell-cell interactions. RNA was extracted from dispersed animal cap ectoderm, either reagggregated after 0, 4, or 7 hours, or maintained in dispersion until stage 15.
- Figure 2. Activin does not induce epidermis. Late blastula animal caps were disaggregated, cultured for 4 hours in various concentrations of activin, and reagggregated. RNA was extracted for RT-PCR at the end of neurulation. In the absence of activin and at low activin concentration dispersed cells are neuralized, expressing NCAM (lanes 2-4). At higher activin concentrations mesoderm is induced, as shown by the presence of Xbra message. At no concentration is epidermis formed. Cells reagggregated immediately after dispersal go on to express epidermal keratin (lane 1), as do intact caps, which also express Xbra when cultured in activin. The activin used in this experiment is partially purified supernatant from P388D1 cells, units are arbitrary. Dispersed cells were cultured in 1X calcium and magnesium-free MBSH with 0.5 mg/ml USA.

Figure 3. BMP-4 induces epidermis in dispersed ectoderm. Dissociated stage 9 animal cap cells as well as intact caps were incubated for 4 hours with or without BMP-4 protein. In the absence of added factor, dispersed cells express NCAM after reaggregation and culture to stage 18 (lanes 2). In contrast, dispersed cells exposed to BMP-4 at all concentrations tried in this experiment strongly expressed the epidermis-specific keratin (3-6). NCAM expression is suppressed. A high concentration of BMP-4 induces the posterior mesodermal marker Xbra in intact caps (lane 9). Recombinant human BMP-4 was obtained from Genetics Institute.

Figure 4. A dominant-negative activin receptor can block the induction of epidermis by BMP-4. Animal caps from embryos previously injected with RNA encoding the truncated activin receptor (Δ 1AR1) and uninjected control embryos were cut and dissociated at the late blastula stage. Dispersed blastomeres were incubated for 4 hours in the presence or absence of 50 ng/ml BMP-4 protein, reaggregated, and cultured to late neurula stage. (A) BMP-4 suppresses neural development and induces epidermis in dispersed ectoderm from control embryos (lane 3), but not in cells from injected embryos, which continue to strongly express NCAM (lane 6). (B) In the same experiment, intact animal caps expressing the truncated receptor expressed NCAM (lane 11). The induction of mesoderm by activin in intact caps was partially blocked by the truncated receptor (lane 12). RNA for injection was synthesized *in vitro* by standard methods; a total of 2 ng of RNA was injected into the animal hemisphere of two cell stage embryos. The construction of Δ 1AR1 was described previously.

Figure 5. Follistatin protein cannot block the epidermalizing activity of BMP-4. Dispersed late blastula ectoderm was cultured in media containing 50 ng/ml BMP-4 protein alone, 1 μ g/ml follistatin protein alone, both proteins at these concentrations, or in the absence of either factor. (A) Prolonged dispersal results leads reaggregated ectoderm to express NCAM (lane 2); exposure to follistatin alone has no effect (lane 3). BMP-4 suppresses NCAM and induces epidermal keratin (lane 4). Follistatin protein, even at a high dose, is unable to block

epidermalization by BMP-4 (lane 5). Figure 5B demonstrates that the follistatin protein used in this experiment was active, completely blocking mesoderm induction by activin in intact caps, as assayed by expression of muscle actin. The dose required to block activin here was 10 ng/ml, 100-fold less than the dose used on dispersed cells. Human recombinant short-form follistatin protein was obtained from NIH.

Figure 6. Neural induction in P19 cells transfected with the truncated *Xenopus* activin type II receptor. P19 cells were stably transfected with the control plasmid, pcDNA3 (A and B) or Δ 1XAR1myc (C and D). Immunofluorescence using a monoclonal antibody to NF-160 as a marker of neural differentiation with a Texas-red conjugated secondary antibody (A and C) and the corresponding DIC micrographs (B and D) are shown. Neural induction was observed in the P19 cell line which was transfected with the truncated receptor.

Figure 7. Colocalization of the Δ 1XAR1myc receptor with neural antigen NF200. The P19 Δ 1XAR1myc cell line was grown on chamber slides and was analyzed for expression of the neural marker NF-200 using a polyclonal antibody and the truncated activin receptor with the myc tag as a marker using the monoclonal antibody, 9E10. Confocal micrographs are shown illustrating indirect immunofluorescence of NF-200 with a fluorescein conjugated secondary antibody (A), the myc tag with a Texas red conjugated secondary antibody (B) and double immunofluorescence of both NF-200 and myc (C). The myc labeling indicates that the truncated activin receptor is expressed in this cell line. In addition, high levels of myc labeling is coincident with neural differentiation.

Figure 8. Time course of neural induction in P19 Δ 1XAR1myc. The cell line P19 Δ 1XAR1myc and a control cell line P19pcDNA3 were plated at low density and neuralization was followed over 7 days using expression of the NF-160 as a marker of neural differentiation. Using indirect immunofluorescence, the number of cells expressing the neural NF-160 antigen was calculated as a percentage of the

total number of cells present in a randomly chosen field of view. The results presented represent averages of approximately 15 fields of view from 2 separate experiments. No neurons were observed in P19pcDNA3 (squares) during the 7 days. In P19 Δ 1XAR1myc (triangles) differentiated neurons were not apparent until the cells had neared confluence which occurred at about day 4.

Figure 9. BMP4 inhibits neuralization of P19 cells by retinoic acid. P19 cells were grown for 4 days on a solid surface in control media (A and B) or media supplemented with 3.6nM BMP4 (C and D), 0.5mM RA (E and F), 0.5mM retinoic acid and 0.36nM BMP4 (G and H) or 0.5mM RA and 3.6nM BMP4 (I and J), followed by 2 days in control media. Neuralization was assessed by indirect immunofluorescence for expression of NF-160 (A, C, E, G, and I). The corresponding DIC pictures are shown (B, D, F, H and J). BMP4 inhibits RA induced neural differentiation at concentrations as low as 0.36nM. This inhibition is more severe when 3.6nM BMP4 is used to challenge RA.

Figure 10. Induction of keratins by BMP4 in P19 cells. P19 cells were grown on solid surfaces for 4 days in control media (A and B) or in media supplemented with 3.6nM BMP4 (C-H) followed by 2 days in control media. Epithelial differentiation was assessed by indirect immunofluorescence for expression of keratin using a mixture of monoclonal antibodies (A, C, E and G). The corresponding DIC pictures are shown (B, D, F and H). Cultures incubated with BMP4 showed keratin expression. Panels C, E and G demonstrate the different cell morphologies observed in keratin expressing cells.

Figure 11. Construct pSP64TS. (A) A schematic of the pSP64T construct (3kb) containing a procaryotic promoter (SP6) and a 30bp insert. The construct provides additional cloning sites, including a blunt end site and stop codons at all three reading frames downstream of the BglII, EcoRV and the SpeI site. The host strain HB101, is ampicillin-sensitive, the plasmid confers ampicillin-resistant. (B) The partial nucleotide sequence of the pSP64TS construct between the HndIII site

and the EcoR1 site showing the 30 bp insert (heavy underlining) in the sense orientation, relative to the promoter.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
OF THE INVENTION

5

More particularly, the present invention relates to a gene product of a member of the TGF- β gene family, and preferably to BMP-4, a peptide growth factor related to activin, which can strongly suppress autoneuralization and induce epidermis in dispersed cells. This induction is blocked by a dominant-negative form of the
10 activin receptor, suggesting that the previously reported neuralizing activity of the truncated receptor in embryos may result from antagonizing endogenous BMP-4. Since BMP-4 is expressed in the *Xenopus* ectoderm at the stage when the decision between epidermal and neural fates is made, this factor appears to be an
15 endogenous neural inhibitor and epidermal inducer, the first reported in any vertebrate. Moreover the discovery of an epidermal inducer lends considerable support to the default model of neural specification.

In accordance with the present invention there may be employed conventional
20 molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*,
25 Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M.
30 Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

The terms "gene product of a member of the TGF- β gene family", "TGF- β -like protein", and other variants not specifically listed, and in a preferred embodiment, "bone morphogenesis protein", "bone morphogenesis factor", "BMP-4", "BMP-2B", "morphogenesis factor", "morphogenesis factor protein(s)", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and active fragments or cognate molecules thereof, and extends to those proteins having the amino acid sequence data described in U.S. Patent No. 5,166,058, which is incorporated herein in its entirety. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "bone morphogenesis protein", "bone morphogenesis factor", "BMP-4", "BMP-2B", "morphogenesis factor", and "morphogenesis factor protein(s)" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

5

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change.

Preferably, the transforming DNA should be integrated (covalently linked) into
10 chromosomal DNA making up the genome of the cell.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

15

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of
20 ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA
25 molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein
30 according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a

sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook et al., *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m , *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m , *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook et al., *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook et al., *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; more preferably at least about 15 nucleotides; most preferably the length is at least about 20 nucleotides.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site

(conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

- 5 A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.
- 10 A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can
- 15 be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

The term "oligonucleotide", as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides or

20 deoxyribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

A "nucleotide probe" as used herein refers to an oligonucleotide of at least about 9

25 bases, which has a sequence corresponding to a portion of the DNA coding for a protein of the TGF- β gene family.

As used herein, the term "sequence homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin,"

30 including proteins from superfamilies (e.g., the immunoglobulin superfamily) and

homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667).

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that do not share a common evolutionary origin (*see* Reeck et al., *supra*).

Two DNA sequences are "substantially homologous" or "substantially similar" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, *e.g.*, Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 70% of the amino acids are identical, or functionally identical. Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program.

The term "corresponding to" is used herein to refer to similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. For example, as demonstrated in Wozney, 1993, *infra*, the sequences of the BMP proteins can be aligned, and the corresponding amino acid residues determined, despite the deletion of amino acid residues at some positions in one BMP protein compared to another. Thus, the

term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. An "antibody combining site" or "antigen recognition site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen. The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v), which portions are preferred for use in the therapeutic methods described herein. The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for

immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al., *Immunology, Second Ed.*, 1984, Benjamin/Cummings: Menlo Park, California, p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc.) when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, which is substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like,

when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

The term "biological sample" is used herein to refer to a sample containing cells that express or may express a gene product of the TGF- β gene family. Such cells may be obtained from a subject, or from *in vitro* culture. The term "biological sample" further extends to an extract of cells from either source.

The term "about" is used herein to mean within a 10% variance from the figure given, preferably within a 5% variance, and more preferably within a 1% variance.

Thus, in instances where it is desired to reduce or inhibit the gene activity resulting from a particular stimulus or factor, an appropriate antagonist of the

binding domain of a gene product of a member of the TGF- β gene family, and specifically of BMP-4 or another TGF- β -like protein could be introduced to block the interaction of the BMP-4 protein with its binding site.

- 5 As discussed earlier, the antagonists of the TGF- β -like protein binding, and specifically BMP-4 binding, may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient experiencing an adverse medical condition associated specific transcriptional stimulation for the treatment thereof. Preferably, the
- 10 pharmaceutical formulation will provide for transmembrane migration of the antagonists, which will be active in the cytoplasm. A variety of administrative techniques may be utilized, among them parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like. Average quantities of the recognition factors or their subunits may vary and
- 15 in particular should be based upon the recommendations and prescription of a qualified physician or veterinarian.

- Also, antibodies including both polyclonal and monoclonal antibodies, may possess certain diagnostic or therapeutic (inhibitory) applications and may for example, be
- 20 utilized for the purpose of detecting and/or measuring conditions such as cellular activation as a result of viral infection, inflammation, or the like. For example, TGF- β -like protein binding domain or specifically the BMP-4 protein binding domain, or a peptide corresponding to a TGF- β -like protein epitope or a BMP-4 protein epitope may be used to produce both polyclonal and monoclonal antibodies
- 25 to themselves in a variety of cellular media, by such well known techniques as immunization of rabbit using Complete and Incomplete Freund's Adjuvant and the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells, respectively. Preferably, such proteins are conjugated to a carrier molecule, as described above. These techniques have been described in numerous
- 30 publications in great detail, *e.g.*, International Patent Publication WO 93/19179, and do not bear repeating here.

Likewise, small molecules that mimic or antagonize the activity(ies) of the TGF- β -like or specifically the BMP-4 protein of the invention may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

- 5 Identification of important regions of the TGF- β -like, or specifically, the BMP-4 proteins for function provides a basis for screening for drugs capable of specific interaction with the functionally relevant domains. Accordingly, in addition to rational design of compounds that bind to, and preferably competitively inhibit the functional activity of the TGF- β -like or BMP-4 protein, *i.e.*, antagonists, based on
- 10 the structure of the relevant domain, the present invention contemplates an alternative method for identifying specific binding compounds of the binding domain or the region containing a biologically active region using various screening assays known in the art.
- 15 Any screening technique known in the art can be used to screen for TGF- β -like or BMP-4 binding antagonists. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and antagonize TGF- β -like proteins or BMP-4 activates *in vivo*.
- 20 Knowledge of the primary sequence of the TGF- β -like protein or BMP-4 binding domain, and the similarity of that sequence with proteins of known function, can provide an initial clue as the inhibitors or antagonists of the protein. Identification and screening of antagonists is further facilitated by determining structural features
- 25 of the protein, *e.g.*, using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.
- 30 The screening can be performed directly using peptides corresponding to the receptor binding domain. Alternatively, chimeric proteins, which contain the

binding domain may be used, as such proteins will contain the element specifically under investigation.

The reagents that contain the TGF- β -like protein or BMP-4 receptor binding
5 domain can be labeled for use in the screening assays. In one embodiment, the compound may be directly labeled. In another embodiment, a labeled secondary reagent may be used to detect binding of the compound to a solid phase support containing a binding molecule of interest. Binding may be detected by *in situ*
formation of a chromophore by an enzyme label. Suitable enzymes include, but
10 are not limited to, alkaline phosphatase and horseradish peroxidase. Other labels for use in the invention include colored latex beads, magnetic beads, fluorescent labels (e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, Lucifer Yellow, AMCA blue, free or chelated lanthanide series salts, especially Eu^{3+} , to name a few fluorophores), chemiluminescent molecules,
15 radio-isotopes, or magnetic resonance imaging labels.

As suggested earlier, the diagnostic method of the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of a reagent that specifically binds to a TGF- β -like or BMP-4
20 protein. Preferably, such a reagent is an antibody, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the anti-BMP-4 antibody molecules used herein be in the form of Fab, Fab', F(ab')_2 or F(v) portions or whole antibody molecules. As previously discussed, patients capable of benefiting from this method include those suffering from
25 wounds, burns, tumor derived from neural tissue or other like pathological derangement. Methods for determining and optimizing the ability of anti-BMP-4 antibodies to assist in the examination of the target cells are all well-known in the art.

30 The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic

composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more agonists or antagonists of a TGF- β -like protein, or specifically BMP-4, *e.g.*, a molecule that specifically interacts with the receptor binding domain of a TGF- β -like or BMP-4 protein, as described herein as an
5 active ingredient.

A gene encoding a mutant TGF- β -like or BMP-4 protein, whether genomic DNA or cDNA, can be isolated from any source, particularly from a human cDNA or genomic library, and mutated according to standard methods. Specific cDNA
10 sequences encoding BMP proteins are disclosed in U.S. Patent Nos. 5,013,649 and 5,166,548, which are incorporated herein by reference in their entirety. Methods for obtaining the BMP gene are well known in the art, as described above (*see, e.g.*, Sambrook et al., 1989, *supra*). Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis
15 (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB[®] linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (*see* Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications*
20 *for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6; pp. 61-70).

Accordingly, any animal cell potentially can serve as the nucleic acid source for the molecular cloning of a TGF- β -like or BMP gene. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA
25 "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (*See, for example, Sambrook et al., 1989, supra; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II*). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to
30 coding regions; clones derived from cDNA will not contain intron sequences.

Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

The nucleotide sequence coding for a mutant TGF- β -like or BMP-4 protein, can
5 be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein "promoters." Thus, the nucleic acid encoding the mutant TGF- β -like or BMP-4 protein of the invention is operatively associated with a promoter in an expression vector of the invention.
10 Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a
15 recombinant expression vector, or they may be supplied by the native gene encoding a TGF- β -like protein or BMP-4 and/or its flanking regions.

In another embodiment, a chimeric TGF- β -like or BMP-4 protein or mutant TGF- β -like or BMP-4 protein can be prepared, *e.g.*, a glutathione-S-transferase (GST)
20 fusion protein, a maltose-binding (MBP) protein fusion protein, or a poly-histidine-tagged fusion protein, for expression in bacteria. Expression of a BMP-4 protein as a fusion protein can facilitate stable expression, or allow for purification based on the properties of the fusion partner. For example, GST binds glutathione conjugated to a solid support matrix, MBP binds to a maltose matrix, and poly-
25 histidine chelates to a Ni-chelation support matrix. The fusion protein can be eluted from the specific matrix with appropriate buffers, or by treating with a protease specific for a cleavage site usually engineered between the TGF- β -like or BMP-4 polypeptide and the fusion partner (*e.g.*, GST, MBP, or poly-His). Furthermore, the present invention contemplates fusions between a domain from
30 one TGF- β -like or BMP-4 protein in the site of the corresponding domain of a second TGF- β -like or BMP-4 protein.

Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast
5 containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

10 A recombinant mutant or chimeric TGF- β -like protein or BMP-4 of the invention, or functional fragment, derivative or analog thereof, may be expressed chromosomally, after integration of the protein coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

15 The cell into which the recombinant vector comprising the nucleic acid encoding the mutant or chimeric TGF- β -like protein or BMP-4 is cultured in an appropriate cell culture medium under conditions that provide for expression of the protein by the cell.

20 Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA
25 and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of a protein may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control gene expression
30 include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long

terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as

5 the β -lactamase promoter (Villa-Kamaroff, et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase)

10 promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals.

Vectors are introduced into the desired host cells by methods known in the art,

15 *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, *J. Biol. Chem.* 267:963-967; Wu and Wu, 1988, *J. Biol. Chem.* 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

20 In one embodiment, a gene encoding a mutant TGF- β -like or BMP-4 protein is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus

25 (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a particular locus, *e.g.*, the organ implicated in the rejection episode, can be

30 specifically targeted with the vector. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt et al., 1991,

Molec. Cell. Neurosci. 2:320-330), an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. (1992, *J. Clin. Invest.* 90:626-630), and a defective adeno-associated virus vector (Samulski et al., 1987, *J. Virol.* 61:3096-3101; Samulski et al., 1989, *J. Virol.* 63:3822-3828).

5

Alternatively, the vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a protein (Felgner, et. al., 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417; see Mackey, et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:8027-8031). The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner and Ringold, 1989, *Science* 337:387-388). The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting (see Mackey, et. al., 1988, *supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, *e.g.*, Wu et al., 1992, *J. Biol. Chem.* 267:963-967; Wu and Wu, 1988, *J. Biol. Chem.* 263:14621-14624;

Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

The preparation of therapeutic compositions which contain polypeptides, analogs
5 or active fragments as active ingredients is well understood in the art. Typically,
such compositions are prepared as injectables, either as liquid solutions or
suspensions, however, solid forms suitable for solution in, or suspension in, liquid
prior to injection can also be prepared. The preparation can also be emulsified.
The active therapeutic ingredient is often mixed with excipients which are
10 pharmaceutically acceptable and compatible with the active ingredient. Suitable
excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like
and combinations thereof. In addition, if desired, the composition can contain
minor amounts of auxiliary substances such as wetting or emulsifying agents, pH
buffering agents which enhance the effectiveness of the active ingredient.

15

A polypeptide, analog or active fragment can be formulated into the therapeutic
composition as neutralized pharmaceutically acceptable salt forms.
Pharmaceutically acceptable salts include the acid addition salts (formed with the
free amino groups of the polypeptide or antibody molecule) and which are formed
20 with inorganic acids such as, for example, hydrochloric or phosphoric acids, or
such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed
from the free carboxyl groups can also be derived from inorganic bases such as,
for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and
such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol,
25 histidine, procaine, and the like.

The therapeutic polypeptide-, analog- or active fragment-containing compositions
are conventionally administered intravenously, as by injection of a unit dose, for
example. The term "unit dose" when used in reference to a therapeutic
30 composition of the present invention refers to physically discrete units suitable as
unitary dosage for humans, each unit containing a predetermined quantity of active

material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition or neutralization of epidermal induction or neuralization inhibition desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

The therapeutic compositions may further include an effective amount of the factor/factor synthesis promoter antagonist or analog thereof, and one or more of the following active ingredients: an antibiotic, a steroid. Exemplary formulations are well known in the art, *e.g.*, as disclosed in International Patent Publication WO 93/19179.

25

An assay useful and contemplated in accordance with the present invention is known as a "cis/trans" assay. Briefly, this assay employs two genetic constructs, one of which is typically a plasmid that continually expresses a particular receptor of interest when transfected into an appropriate cell line, and the second of which is a plasmid that expresses a reporter such as luciferase, under the control of a receptor/ligand complex. Thus, for example, if it is desired to evaluate a

30

compound as a ligand for a particular receptor, one of the plasmids would be a construct that results in expression of the receptor in the chosen cell line, while the second plasmid would possess a promoter linked to the luciferase gene in which the response element to the particular receptor is inserted. If the compound under
5 test is an agonist for the receptor, the ligand will complex with the receptor, and the resulting complex will bind the response element and initiate transcription of the luciferase gene. The resulting chemiluminescence is then measured photometrically, and dose response curves are obtained and compared to those of known ligands. The foregoing protocol is described in detail in U.S. Patent No.
10 4,981,784 and PCT International Publication No. WO 88/03168, for which purpose the artisan is referred.

In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of
15 predetermined transcriptional activity or predetermined transcriptional activity capability in suspected target cells, as set forth above. In accordance with the testing techniques discussed above, one class of such kits will contain at least a reagent capable of specifically binding to the TGF- β -like or BMP-4 protein, and means for detecting binding of the reagent to a TGF- β -like or BMP-4 protein. In
20 a specific aspect, such a reagent is an antibody. Means for detecting binding may be a label on the antibody (labels have been described above), or a label on a TGF- β -like or BMP-4 protein or fragment thereof. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

25 The present invention begins with the observation that *Xenopus* ectoderm cultured in dispersion during early gastrula stages forms neural tissue. Cells dispersed for three hours or more, whether subsequently reaggregated or not, express the general neural marker NCAM at neurula stages. The present results confirm those of (1) Grunz, who found neural structures in animal caps subjected to prolonged
30 dissociation; (2) Sato and Sargent, who reported NCAM expression in disaggregated whole embryos; and (3) Godsave and Slack, who showed that

cultures of individual animal pole blastomeres often gave rise to neurons. As Godsave and Slack were the first to point out, this phenomenon suggests that individual ectoderm cells are neurally specified at the start of gastrulation, yet intact animal caps explanted at this stage develop as epidermis in the absence of added factors. It follows that intact ectoderm must contain an activity responsible for imposing and maintaining epidermal fate, an activity which is lost, presumably by dilution, when the cells are dispersed.

Moreover, since caps dispersed and immediately reaggregated also form epidermis, the epidermalizing signals appear to be produced and secreted by the cells themselves during gastrula stages. (In this respect, epidermal specification of the ectoderm formally resembles the class of phenomena grouped under the term "community effect").

The present invention demonstrates that the soluble growth factor BMP-4 can induce epidermis in dispersed cells and suppress auto-neuralization. Ectodermal cells exposed to the BMP-4 express epidermal keratin and NCAM expression is suppressed. BMP-4 can exert this effect at low concentrations, below those needed for other reported activities of this protein. This is the first report of epidermal induction in vertebrates by a defined factor.

BMP-4 can induce epidermis, and recent work establishes that it meets another crucial test: it is expressed at the appropriate time and place. *In situ* hybridization, carried out by Fainsod et al and by Brivanlou and Thomsen, shows that BMP-4 RNA is present in the entire animal cap at the start of gastrulation, as well as in ventral and lateral marginal zone. At later stages, the transcript disappears from the portion of the ectoderm that becomes the neural plate. Thus the pattern of BMP-4 transcription is consistent with a function in the induction of epidermis and the suppression of neural development.

The present invention demonstrates that interference with TGF- β signaling in a mammalian embryonic cell line leads to the specification of neural cells, indicating that the molecular strategy behind neural determination is conserved throughout vertebrate evolution. Disruption of signaling by stable expression of a truncated,
5 dominant-negative, *Xenopus* activin type II receptor (Δ IXAR1myc) can induce neurons in P19 cells. In addition, the present invention demonstrates that BMP4 can inhibit the RA induced neural differentiation of P19 cells and is capable of inducing epithelial cells in P19 cultures as assessed by keratin expression.

10 The present invention also demonstrates that a related protein, activin, cannot epidermalize in the dispersed cell assay. However, there are other plausible candidates that have not been tested, since the TGF- β family is large and growing. In particular, the other BMPs are likely alternatives. BMP-7 deserves special attention, since it too is expressed in the gastrula ectoderm.

15

The present invention also demonstrates that, as in *Xenopus* embryos and embryonic explants, a truncated activin type II receptor neuralizes P19 cells; and that the mechanism previously proposed, in which inhibition of TGF- β signaling is required for neural differentiation, is evolutionarily conserved from amphibians to
20 mammals.

In *Xenopus*, the truncated activin receptor induces neurons in the absence of mesoderm induction (Hemmati-Brivanlou and Melton, 1994). In the context of the P19 cells, neural specification in the apparent absence of mesoderm was observed
25 since both skeletal and cardiac myosin antigens were not expressed in the transfected cells. Assessing the lack of mesoderm in this cell line is complicated, however, by the fact that the *brachyury* gene, a mesodermal marker, is expressed at low levels in undifferentiated P19 cells. *Brachyury* is induced when P19 cells undergo mesodermal differentiation in response to DMSO but not during neural
30 differentiation by RA (Vidricaire *et al.*, 1994). In addition, induction of *brachyury* mRNA by activin A has been reported (van den Eijnden-van Raaij *et*

al., 1991; Vidricaire *et al.*, 1994). These observations parallel those made in *Xenopus* in which *brachyury* (*Xbra*) is induced by activin, although, contrary to *Xenopus*, activin does not induce mesodermal cell types such as muscle in P19 cells. No increased *brachyury* expression in the P19 Δ 1XAR1myc cell line as
5 compared to undifferentiated P19 cells was observed, indicating that mesoderm induction is not a prerequisite for neural differentiation.

Not all cells in the P19 Δ 1XAR1myc cell line differentiate into neurons. Variable expression within the clonal cell line could be the result of differential activation
10 of the CMV promoter among cells. Similar experiments in which mNotch was stably expressed in P19 cells resulted in variable levels of expression of the mNotch construct within a line (Nye *et al.*, 1994). In addition, cell lines expressing high levels of Δ 1XAR1 would be expected to contain more differentiated cells, making it difficult to isolate a line, since cells which
15 differentiate exit the cell cycle. The line isolated may express the Δ 1XAR1myc construct at low enough levels to permit maintenance of the cell line but with sufficient promoter variability to generate higher levels of expression in a few cells. Two lines expressing the mouse truncated activin type II receptor under the control of a metallothionien promoter were isolated. These lines displayed a
20 marked increase in the number of neurons in the presence of zinc.

The truncated type II activin receptor inhibits all BMPs and activins tested so far (Hemmati-Brivanlou and Thomsen, 1995; Kessler and Melton, 1995; Schulte-Merker *et al.*, 1994). Thus any of these factors can be a potential
25 candidate for the endogenous neural inhibitor. Recent knockout experiments in the mouse has helped in providing clues about the factors acting in the early embryo. Elimination of both activin A and B from the embryo has no consequence in the establishment of ectodermal patterning and neural fate specification (Matzuk *et al.*, 1995b; Vassalli *et al.*, 1994), although these results can be explained by either the
30 possible necessity for a maternal source of activin A or overlapping function from additional activin ligands such as the recently discovered activin C and D (Hötten

et al., 1995; Oda *et al.*, 1995). Also mice deficient in the type II activin receptor, ActRcII, have normal neural development and ectodermal patterning (Matzuk *et al.*, 1995a). Here also, parallel signaling from additional activin type II receptors cannot be excluded. However, the knockouts of BMP4 ligand or the type I
5 receptor (BMPR) results in embryonic death at gastrulation (Mishina *et al.*, 1995; Winnier *et al.*, 1995), the time when ectodermal patterning occurs. This final observation taken together with the fact that dominant negative BMP4 ligand and receptor induce direct neuralization in ectodermal explants (Graff *et al.*, 1994; Suzuki *et al.*, 1994; Xu *et al.*, 1995) and that BMP4 can act as an epidermal
10 inducer in *Xenopus* embryos (Wilson and Hemmati-Brivanlou, 1995), points to BMP4 as the ligand to act in cell fate specification in the context of the ectoderm *in vivo*.

The molecular pathway involved in the establishment of different cell fates within
15 the embryonic ectoderm shares intriguing similarities between the fruit fly and the frog. BMP4 is the vertebrate homolog of the *Drosophila* TGF- β family member *dpp*, and *chordin* is the vertebrate counterpart of short gastrulation (*sog*). In both the fruit fly and the frog antagonistic interactions between either BMP4 and *chordin* or *dpp* and *sog* are responsible for ectodermal patterning and the
20 establishment of the neural and epidermal fate.

The present invention indicates that the same molecular mechanisms are operating during arthropod, amphibian and mammalian development and that the conservation of the molecular pathway involved in ectodermal cell fate decision
25 extends beyond arthropods and amphibians to include amniotes.

As Brivanlou and Melton proposed earlier, neuralization appears to involve inhibition of the epidermal inducer. Since simple dispersion in the absence of added factors results not only in the repression of epidermal differentiation but in
30 the formation of neural tissue, such a block is apparently sufficient as well as necessary for neural specification. Two experimental treatments known to

neuralize animal caps plausibly act by this route: (1) injection of RNAs encoding a truncated, dominant-negative form of the activin receptor; and (2) follistatin.

Although these interventions were designed to interfere with activin signalling, both may act against BMP-4. It has recently been demonstrated that the truncated
5 activin receptor can block the induction of ventral mesoderm by BMP-4 in intact animal caps, and the present invention demonstrates that it can also prevent epidermalization by BMP-4 in dispersed cells.

Follistatin, however, is unable to block BMP-4 in the assay of the present
10 invention, even^d at quite high concentrations. It may be that follistatin is not an endogenous neural inducer, perhaps serving instead to limit the spread of mesoderm induction by activin. Injected follistatin RNA, may neuralize the animal cap by blocking an earlier activin-requiring process necessary for later epidermal specification. This may explain why it has not been shown that
15 follistatin can neuralize when supplied at gastrula stages, either as a purified protein or by injecting a plasmid containing follistatin driven by a gastrula-stage promotor (data not shown). Finally, follistatin may in fact block BMP-4 signalling in the embryo although it is unable to do so in the context of dispersed ectoderm. This could be, for example, because follistatin-BMP-4 interaction requires a third
20 player, perhaps activin.

Several molecules in addition to follistatin have recently been shown to neuralize *Xenopus* ectoderm, including the secreted protein noggin and a truncated versions of the transcription factors Brachyury and Xlim. Conceivably these treatments
25 might interfere with BMP signalling by blocking signal transduction, or by repressing BMP transcription. BMP-4 transcripts disappear from the prospective neural plate during gastrulation.

BMP-4 may act in the ectoderm to suppress a so-called default fate. When this
30 signalling is blocked, by injection of a dominant-negative receptor, an unsuspected

dorsal specification is revealed. Thus it appears that BMP-4 may play a similar role in the marginal zone and the animal cap.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

10 Animal cap ectoderm is neuralized by prolonged dispersal

Several groups have reported the neuralization of late blastula or early gastrula ectoderm by prolonged dispersal. In particular, Grunz found observed archencephalic (forebrain) structures in animal caps dissociated for several hours. The present invention confirms this finding, using molecular markers to add to

15 Grunz' histological characterization. Figure 1 A shows that when late blastula animal caps are disaggregated and cultured in dispersion for several hours before being allowed to reaggregate, the general neural marker NCAM is subsequently expressed. Furthermore, the epidermal keratin XK81, strongly expressed in intact animal caps, is turned off by prolonged dispersion. Note that ectoderm

20 reagggregated immediately after dispersal forms epidermal rather than neural tissue, implying that disaggregation itself is not sufficient to neuralize. In agreement with Grunz, it has been found that at least two to three hours of dispersed culture are necessary. Epidermal keratin levels are lower in immediately reagggregated explants than in intact caps because this marker is most strongly expressed in the

25 outer layer of the ectoderm, which is discarded during disaggregation. Thus it is confirmed that animal cap ectoderm cultured during early gastrula stages as dispersed cells forms neural tissue rather than epidermis, despite the absence of dorsal mesoderm the source of neural inducing signals in the embryo.

30 NCAM expression by dispersed animal cap cells does not require that the ectoderm be reagggregated (Figure 1B). This implies that neural specification does

not result from cell-cell interactions taking place after the period of dispersal, as it would, for example, if dispersal somehow rendered some cells capable of neuralizing their neighbors.

5

EXAMPLE 2

Activin cannot restore dispersed ectoderm to an epidermal fate.

If isolation of early gastrula ectoderm from intercellular signals leads to neural differentiation, it follows that the epidermal specification of intact explants, and of
10 the ventral ectoderm of the embryo, must result from signalling within the ectoderm. Moreover, the neuralizing effects of follistatin and the dominant negative form of the activin receptor point to the secreted growth factor activin, or a close relative, as the epidermal inducer. To test this hypothesis, various concentrations of activin (in the form of a partially purified supernatant from an
15 activin-producing cell line) were added to dispersed ectodermal cultures. The results of such an experiment are shown in Figure 2. At the lower concentrations activin had no effect: after reaggregation and culture, cells exposed to these levels expressed NCAM and turned off the epidermal marker, just as in the absence of activin. As expected from earlier work, higher concentrations of activin induced
20 the dispersed ectoderm to form mesoderm, revealed here by expression of the mesodermal marker Xbra. (Even higher doses of activin would have prompted the expression of more dorso-anterior mesodermal markers.) Both NCAM and epidermal keratin are turned off in these mesodermalized reagggregates. At no concentration of activin was epidermis formed. Since it might be argued that an
25 epidermis-inducing dose of activin lay in between the doses used here, this experiment was repeated using concentrations separated by only 25%. Again, no epidermis was formed at any activin concentration (data not shown). Moreover, in this experiment, one dose of activin proved to be right at the transitional dose, such that some cells were induced to form mesoderm while other continued to
30 express NCAM. (In Figure 2 as well, some NCAM expression persists at 1.0 units of activin, where xbra is already strongly expressed (lane 5). This argues

persuasively against the existence of an epidermis-inducing activin concentration below the mesoderm threshold. Thus, in this assay, activin is not able to substitute for an endogenous epidermal inducer inactivated by the dispersal of gastrula ectoderm. Activin is able to inhibit the neuralization of dispersed cells,
5 but only by inducing mesoderm, not epidermis.

EXAMPLE 3

**The dominant-negative activin receptor, but not follistatin protein, can block
10 epidermal induction by BMP-4.**

Both a truncated, dominant-negative form of the type II activin receptor ($\Delta 1XAR1$) and the activin antagonist follistatin have been shown to neuralize animal caps, when expressed from injected synthetic RNAs. Since these treatments were presumed to exert their effects by blocking the action of an
15 endogenous epidermal inducer and neural inhibitor, experiments were performed to determine whether these agents could block the effect of BMP-4 on dispersed cells. The dominant negative receptor was tested by applying BMP-4 protein to dispersed ectoderm from embryos previously injected with $\Delta 1XAR1$ RNA. Figure 4A demonstrates that the truncated receptor can block epidermalization by BMP-4:
20 cells from uninjected caps are induced to express epidermal keratin, but those from injected embryos continue to express NCAM after exposure to BMP-4 protein. As expected from previous work, intact caps containing truncated receptor were neuralized, and mesoderm induction by activin was inhibited, although only partially at the concentrations used in this experiment (Figure 4B).

25 The effect of follistatin was examined using soluble protein rather than injected RNA, since protein produced by injected cells would be diluted by dispersal. Late blastula ectoderm was dispersed as before and cultured for four hours in media containing BMP-4, BMP-4 and follistatin protein, or neither factor. As Figure 5A
30 shows, follistatin is apparently unable to block BMP-4 action in this assay, although in accompanying experiments the same preparation of follistatin is a

potent inhibitor of activin (Figure 5B). Even at a concentration 100 fold greater than that required to block muscle induction by activin (assayed by expression of cardiac actin), follistatin can neither prevent the induction of epidermal keratin nor restore NCAM expression in ectoderm exposed to BMP-4 during dispersed
5 culture. In other experiments, even a 50-fold molar excess of follistatin does not prevent induction of epidermis by BMP-4.

EXAMPLE 4

10 **Inhibitory control of neural differentiation in mammalian cells.**

To test if the TGF- β family members implicated in *Xenopus* could also direct similar cell fate decisions in embryonic mammalian cells, the effects of activin and BMP4 signaling in P19 cells were examined. P19 is a pluripotent mouse embryonic carcinoma cell line capable of differentiating into many cell types. P19
15 cells, when injected into mouse blastocysts, can contribute to many different tissue types indicating that these cells maintain the capacity to differentiate in response to many developmental signals (Rossant and McBurney, 1982). While undifferentiated P19 cells can be stably maintained in culture, various agents can induce differentiation of these cells. Aggregation in the presence of retinoic acid
20 (> 100nM) results in induction of neurons and astroglial cells while aggregation in the presence of DMSO (0.8-1.0%) results in induction of mesodermal cell types such as cardiac and skeletal muscle (reviewed in Bain *et al.*, 1994; McBurney, 1993). Neurons resulting from retinoic acid (RA) induced differentiation maintain many features of normal neurons, such as a neural morphology and the ability to
25 develop functional synapses (Morassutti *et al.*, 1994). In addition, RA induced neuralization of P19 cells is accompanied by the induction of several factors, such as Mash-1, Wnt-1 and mNotch, associated with neural development *in vivo* (reviewed in Bain *et al.*, 1994). These factors appear to follow a temporal pattern during neural differentiation similar to that observed in the mouse embryo. These
30 results support that the basic mechanisms of neural differentiation are intact in

these cells and that P19 cells provide a convenient model for the study of early mammalian neural development.

A Dominant-Negative Activin Receptor Induces P19 Cells to Differentiate into

5 Neurons:

In order to study the role of TGF- β signaling in P19 cells, a truncated form of the *Xenopus* activin type II receptor (Δ IXAR1) was cloned into an expression plasmid, pcDNA3, in which expression is under the control of a CMV promoter. The *Xenopus* receptor was chosen since its inhibitory functions have been well
10 characterized in the context of a developmental system (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994). In addition, activin receptors are capable of functioning across species (Suzuki *et al.*, 1994). To distinguish between the transfected receptor and the endogenous form, an epitope tag, which contains 10 amino acids from the human c-myc gene, was fused to the
15 carboxyl terminus of the truncated receptor. This epitope did not interfere with the function of the receptor in the context of *Xenopus* ectodermal explants (data not shown) and can be recognized by the monoclonal antibody, clone 9E10, without species cross reactivity with endogenous myc proteins (Evan *et al.*, 1985). The truncated activin receptor was stably introduced into the embryonic carcinoma
20 cell line, P19 using a selectable marker, the neomycin resistance gene within the plasmid. Several neomycin resistant P19 lines were isolated. These lines were initially screened for differentiation using the neural specific monoclonal antibody, neurofilament 160 (NF-160, Dubus *et al.*, 1983). While many lines did not express the NF-160 antigen or expressed it at low levels, one line
25 (P19 Δ IXAR1myc) showed more extensive NF-160 expression (Figure 6). This neuralization was observed in P19 cells cultured on tissue culture plates without the requirement of either aggregation or RA treatment. The NF-160 positive cells had a typical neural phenotype with small cell bodies and long branching axons and dendrites (Figure 6C). Cells expressing NF-160 were not observed in stable
30 cell lines transfected with the pcDNA3 vector alone (Figure 6A) demonstrating that the neural cells formed were dependent on the expression of Δ IXAR1myc.

The expression of another neural specific antigen, NF-200 (Dubus *et al.*, 1983) was tested, in P19 Δ 1XAR1myc cells with a polyclonal antibody specific for the antigen. Figure 7 shows that neural cells expressing NF-200 can be easily detected specifically in the P19 line expressing Δ 1XAR1myc. As an independent
5 test for the specificity of the observed phenotype, double immunofluorescence shows that the myc epitope is expressed in the same cells that are expressing the neural specific antigen (Figure 7C). The level of staining for both the myc antibody and the neural specific antibodies is different from cell to cell. Some cells display only low levels of expression while other cells have bright, punctate
10 labeling. The cells displaying a neural phenotype as assessed by NF-200 staining are those that express the highest amount of the truncated receptor, as visualized with the anti-myc antibody (Figure 7B). While much of the myc epitope is observed in the cell bodies, some labeling can be seen in the axons, suggesting that the truncated receptor is expressed throughout the cell membrane. The
15 expression of mesodermal markers such as skeletal and cardiac myosin in P19 Δ 1XAR1myc cells were not detected, indicating that neural induction was not a secondary consequence of mesoderm induction (data not shown). This parallels the observation that *brachyury*, a mesodermal marker, is not induced in response to concentrations of RA required for neural differentiation (Vidricaire *et al.*,
20 1994). An increase of *brachyury* in the P19 Δ 1XAR1myc line when compared to normal P19 cells was not seen (data not shown). Finally, the induction of nervous system cell types was apparently restricted to neurons, since no astroglial cells were detected by an antibody to glial fibrillary acidic protein (GFAP, data not shown).

25

During culturing of this cell line, the percentage of cells displaying the neural antigen NF-160 increased as the cells reached confluence. In the case of RA, it has been noted that cell cell contact is required for the generation of neural fate (Schmidt *et al.*, 1992). In order to assess the importance of cell density in the
30 specification of neural fate, a time course was used in which the cells were plated at low density and cultured for several days without re-plating. No neurons were

observed when cells were plated at low density, as assessed by the expression of the NF-160 antigen. After 4 days in culture, at which time the cells were nearing confluence, some cells (less than 0.1%) began to display a neural phenotype. By day 6, approximately 1.6% of the cells were neurons, and after 7 days in culture, the neural component reached approximately 2.3% (Figure 8). The percentage of cells expressing the neural marker was comparable to day 7 RA treated, aggregated cultures of P19 cells in which 5-10% of the cells were neurons. No neural cells were observed in cells transfected with the vector alone. The appearance of neurons after confluence supports a role for cell-cell contact in neural differentiation in P19 cells.

BMP4 Inhibits Retinoic Acid Induced Neural Differentiation:

Experiments presented above demonstrate that interference with the type II activin receptor can lead to the induction of the neural fate, suggesting that ligands interacting with the receptor act as neural inhibitors. During *Xenopus* gastrulation ectodermal cells have to make a decision between two possible fates: neural on the dorsal side and epidermal in the ventral side. This decision is regulated by a single growth factor, BMP4, which has both neural inhibition and epidermal induction activity in ectodermal cells (Wilson and Hemmati-Brivanlou, 1995). In addition, BMP4 activity can be antagonized by the $\Delta 1XAR1$, making this factor a good candidate for a neural inhibitor (Hemmati-Brivanlou and Thomsen, 1995). In the dorsal side the activity or expression of BMP4 must be inhibited for the neural fate to be unveiled. Because of the observations made in the frog embryo, the next question was whether BMP4 protein can display the same type of activity in the context of the mouse embryonic cell line. Toward this aim, P19 cells were grown on solid surfaces in the presence of RA and pure human BMP4 recombinant protein. Cultures were then examined for neural differentiation using the NF-160 antibody. As previously described (McBurney, 1993), Figure 9E shows that P19 cells grown in the presence of 0.5mM RA showed extensive neural differentiation. Figure 9C shows that BMP4 alone even high concentrations (3.6nM) had no neural inducing ability. When applied together, BMP4 protein

completely blocks neural induction by RA (Figure 9G and I). This inhibition, as manifested by both lower number of cells with NF-160 positive staining cells and lack of neural phenotype, is observed even when BMP4 concentrations as low as 0.36nM are used to challenge the inducing activity of RA, pointing to a physiological effect. The inhibition followed a dose response effect and was even more extensive in the presence of higher concentration of the BMP4 protein (Figure 9I). The amount of BMP4 required to inhibit RA induction is comparable to both the doses required for activin inhibition of RA induced P19 cells (Hashimoto *et al.*, 1990) and to the amount of BMP4 protein required to achieve the same effect in the context of *Xenopus* ectodermal explants (Wilson and Hemmati-Brivanlou, 1995). This inhibition was also observed in cells aggregated in suspension as opposed to grown on solid surfaces indicating that the effect is independent of growth conditions (data not shown). These results demonstrate that BMP4 can inhibit neural differentiation in P19 cells, suggesting that its neural inhibition activity has been conserved in vertebrate evolution.

In *Xenopus*, BMP4 inhibits neural differentiation and induces epidermis in ectodermal cells, suggesting that these two activities represent a single action. Since BMP4 can inhibit neural specification in P19 cells, the question was whether epidermis was induced at the same time. Since no early epidermal specific marker has been described in these cells or in the mouse embryo, an antibody mixture recognizing several keratins that are markers for epithelial cells including epidermis was used (Kopan and Fuchs, 1989; Moll *et al.*, 1982). This mixture contains antibodies against keratins 1, 4, 5, 6, 8, 10, 13, 18 and 19 (Sigma). P19 cultures grown for 6 days on a solid surface without any inducers contained only a few cells which react with the antibody mixture (Figure 10A). Cells grown in the presence of BMP4 showed a dramatic increase in the number of cells expressing the keratin antigens (Figure 10C). At least four different morphologies of keratin expressing cells were observed in these cultures (Figure 10C, E and G). Since the keratin antibodies recognize a mixture of keratins, it is difficult to determine which types of epithelial cells are being produced in response to BMP4. One type of

keratin expressing cells which formed sheets of tightly packed cells with prominent nuclei and diffuse cytoplasmic keratin staining, characteristic of embryonic epidermal cells, was abundant in cultures treated with BMP4 (Figure 10G). As a result, it is clear that BMP4 is capable of inhibiting neural differentiation while
5 inducing epithelial cells.

Vector Construction:

The truncated Xenopus type II activin receptor (Δ 1XAR1) cDNA (Hemmati-Brivanlou and Melton, 1992) was cloned into pSP64TS (Figure 11).

- 10 The resulting carboxyl terminus sequence is GLKPLOPGGSTSSREQKLISEEDL where the sequences in bold represent the myc tag and the underlined sequence originates from the C-terminal end of the truncated activin receptor. This cDNA was subcloned into the HindIII/Not I sites of pcDNA3 (In Vitrogen) in which expression is under the control the CMV promoter. The resulting vector,
15 Δ 1XAR1myc, contains the 5' UTR of the Xenopus β -globin gene followed by the myc-tagged, truncated activin receptor. The poly A⁺ addition site is supplied by bovine growth hormone (BGH) polyadenylation signal within pcDNA3. pcDNA3 also contains a neomycin resistance marker.

20 Cell Culture and Transfection:

- P19 cells were obtained from ATCC and cultured according to Rudnicki and McBurney (Rudnicki and McBurney, 1987). Cells were cultured in α -MEM with 7.5% bovine calf serum and 2.5% fetal calf serum. For retinoic acid (RA) treatment, cells were incubated on solid surfaces in media supplemented with
25 0.5mM all trans retinoic acid (Sigma) for 4 days, with media replacement every 2 days, followed by culture for 2 days in fresh media without RA. Similarly, for BMP4 treatment, cells were incubated on solid surfaces for 4 days in media containing BMP4 or BMP4 and RA at the indicated concentrations, followed by 2 days in media without any factor. For aggregation, P19 cells were grown in
30 suspension in bacterial plates at an initial density of 5×10^5 cells/ml in media with or without factors. Media was replaced every 2 days followed by plating after 4

days onto a solid surface in the absence of factors. Human BMP4 was a gift from Genetics Institute Inc.

For stable transfections, P19 cells were transfected on 100mm plates with plasmid
5 DNA (10mg) using calcium precipitate-mediated transfection (Wigler *et al.*, 1979). Colonies were selected and maintained in media supplemented with 350mg/ml geneticin (Gibco/BRL). Approximately 40 colonies transfected with the myc-tagged truncated activin receptor and 10 colonies transfected with pcDNA3 were isolated. Early passage cells were used in all experiments.

10

Immunofluorescence Analysis:

For immunofluorescence, NF-160 (clone NN18) monoclonal antibody and NF-200 polyclonal antibody were obtained from Sigma and the myc monoclonal antibody, clone 9E10, was obtained from Oncogene Science (Evan *et al.*, 1985). The
15 keratin antibody (Sigma, C 2562) contains a mixture of monoclonal antibodies and will react with keratins 1, 4, 5, 6, 8, 10, 13, 18 and 19. All secondary antibodies, Texas red conjugated, goat, anti-mouse IgG, fluorescein conjugated, goat, anti-mouse IgG and fluorescein conjugated, goat, anti-rabbit IgG were obtained from Jackson Laboratories. Cells were plated and grown on gelatin
20 coated, 8-well, Permanox, chamber slides (LabTek). At the indicated time, cells were washed with PBS, fixed for 10 min. at room temperature in 4% paraformaldehyde made in PBS, washed twice with PBS, permeabilized with methanol for 2 min. at room temperature and washed several times with PBS. Prior to addition of antibody, cells were preincubated in 10% goat serum in PBT
25 (PBS with 2mg/ml BSA and 0.1% triton-X-100) for 1h at room temperature. For primary antibodies, cells were incubated overnight at 4°C in 10% goat serum and PBT followed by 4 washes in PBT. The primary antibodies were used at the following dilutions: NF-160 at 1:80, NF-200 at 1:200 and keratin at 1:100. The 9E10 antibody was used at a final concentration of 2mg/ml. Secondary antibodies
30 at a 1:200 dilution were incubated for 1h at room temperature in 10% goat serum and PBT. Following the secondary antibody, cells were washed 4-5 times with

PBT over 20 min. at room temperature. For Hoechst staining, cells were incubated in Hoechst (1mg/ml in PBS) for 5 min. at room temperature followed by two PBS washes. For visualizing the fluorescence, a Zeiss Axioplan microscope was used with the appropriate filters. Figure 7 was photographed using a Zeiss
5 confocal microscope.

For quantitation of neural phenotype, as detected by the NF-160 was calculated by plating, cells on chamber slides as described above. Total number of cells in each field was assessed by using the Hoechst nuclear staining and neural cells were
10 assessed by the expression of the NF-160 antigen.

The following is a listing of certain of the publications referred to in abbreviated fashion in the foregoing specification.

15

REFERENCES

- Asashima et al. (1990). Roux's Arch. *Dev. Biol.* **198**:645-56.
- Bain, G., Ray, W. J., Yao, M., and Gottlieb, D. I. (1994). From embryonal carcinoma cells to neurons: The P19 pathway. *BioEssays* **16**, 343-348.
- 20 Beddington, R. S. P. (1994). Induction of a second neural axis by the mouse node. *Dev.* **120**:613-620.
- Dawid, I. B. (1994). Intercellular signaling and gene regulation during early embryogenesis of *Xenopus laevis*. *J. Biol. Chem.* **269**:6259-6262.
- Dollé et al. (1989). *Nature* **342**:767-42.
- 25 Dollé et al. (1989). *Nature* **342**:702-705.
- Dubus, E., Weber, K., and Osborn, M. (1983). Monoclonal antibodies specific for glial fibrillary acidic (GFA) protein and for each of the neurofilament triplet polypeptides. *Differentiation* **25**:193-203.
- Evan, G. I., Lewis, G. K., Ransay, G., and Bishop, J. M. (1985). Isolation of
30 monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5**:3610-3616.

- Godsave, S. F., and Slack, J. M. W. (1989). Clonal analysis of mesoderm induction in *Xenopus laevis*. *Development Biology* 134:486-490.
- Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J., and Melton, D. A. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral
5 mesoderm-inducing signals override dorsal signals in vivo. *Cell* 79:169-79.
- Grunz, H., and Tacke, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Diff. Devl.* 28:211-218.
- Harland, R. M. (1994). The transforming growth factor b family and induction of
10 the vertebrate mesoderm: Bone morphogenetic proteins are ventral inducers. *Proc. Natl. Acad. Sci. USA* 91:10243-10246.
- Hashimoto, M., Kondo, S., Sakurai, T., Etoh, Y., Shibai, H., and Muramatsu, M. (1990). Activin/EDF as an inhibitor of neural differentiation. *Biochem. Biophys. Res. Comm.* 173:193-200.
- 15 Hashimoto, M., Nakamura, T., Inoue, S., Kondo, T., Yamamda, R., Eto, Y., Sugino, H., and Muramatsu, M. (1992). Follistatin is a developmentally regulated cytokine in neural differentiation. *J. Biol. Chem.* 267: 7203-7206.
- Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N.,
20 Watabe, T., Blumberg, B. W., and Cho, K. W. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev* 9:2923-35.
- Heine et al. (1987). *J. Cell. Biol.* 105:2861-76.
- Hemmati-Brivanlou, A., Kelly, O. G., and Melton, D. A. (1994). Follistatin, an
25 antagonist of activin is expressed in the Spemann Organizer and displays direct neuralizing activity. *Cell* 77:283-295.
- Hemmati-Brivanlou, A., and Melton, D. A. (1994). Inhibition of activin signaling promotes neuralization in *Xenopus*. *Cell* 77:273-281.
- Hemmati-Brivanlou, A., and Thomsen, G. H. (1995). Ventral mesodermal
30 patterning in *Xenopus* Embryos: Expression patterns and activities of BMP-2 and BMP-4. *Dev. Gen.* 17:78-89.

- Hemmati-Brivanlou, A., and Melton, D. A. (1992). A Truncated Activin Receptor Dominantly Inhibits Mesoderm Induction and Formation of Axial Structures in *Xenopus* Embryos. *Nature* 359:609-614.
- Hötten, G., Neidhardt, H., Schneider, C., and Pohl, J. (1995). Cloning of a new member of the TGF- β family: A putative new activin bc chain. *Biochem. Biophys. Res. Comm.* 206:608-613.
- Hsueh et al. (1987). *Proc. Natl. Acad. Sci. USA* 84::5082-86.
- Immerglück et al. (1990). *Cell* 62:261-68.
- Kessler, D. S., and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* 121:2155-64.
- Kimelman and Kirschner (1987). *Cell* 51:869-77.
- Kintner, C. R., and Dodd, J. (1991). Hensen's node induces neural tissue in *Xenopus* ectoderm. Implications for the action of the organizer in neural induction. *Dev* 113:1495-1506.
- Kondo, S., Hashimoto, M., Etoh, Y., Murata, M., Shibai, H., and Muramatsu, M. (1989). Identification of the two types of specific receptor for activin/EDF expressed on Friend Leukemia and embryonal carcinoma cells. *Biochem. Biophys. Res. Comm.* 161:1267-1272.
- Kopan, R., and Fuchs, E. (1989). A new look into an old problem: keratins as tools to investigate determination, morphogenesis, and differentiation in skin. *Genes Dev.* 3, 1-15.
- Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., and Yancopoulos, G. D. (1993). Neural induction by the secreted polypeptide noggin. *Science* 262:713-718.
- Lee (1990). *Mol. Endoc.* 4:1034-40.
- Lehnert and Akhurst (1988). *Development* 104:263-73.
- Luyten et al. (1989). *J. Biol Chem.* 264:13377-80.
- Lyons et al., 1990, *Development* 109:833-44.
- Lyons et al. (1989). *Proc. Natl. Acad. Sci. USA* 86:455-58.
- Lyons et al. (1989). *Genes Dev.* 3:1657-68.

- Matzuk, M. M., Kumar, T. R., and Bradley, A. (1995a). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* 374:356-360.
- Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R.,
5 Jaenisch, R., and Bradley, A. (1995b). Functional analysis of activins during mammalian development. *Nature* 374:354-356.
- McBurney, M. W. (1993). P19 embryonal carcinoma cells. *Int. J. Dev. Biol.* 37: 135-140.
- Mishina, Y., Suzuki, A., Ueno, N., and Behringer, R. R. (1995). Bmpr encodes a
10 type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes and Development* 15:3027-3037.
- Moll, R., Franke, W. W., and Schiller, D. L. (1982). The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11-24.
- 15 Morassutti, D. J., Staines, W. A., Magnuson, D. S. K., Marshall, K. C., and McBurney, M. W. (1994). Murine embryonal carcinoma-derived neurons survive and mature following transplantation into adult rat striatum. *Neuroscience* 58:753-763.
- Mummery, C. L., and van den Eijnden-van Raaij, A. J. M. (1993). Type b
20 transforming growth factors and activins in differentiating embryonal carcinoma cells, embryonic stem cells and early embryonic development. *Int. J. Dev. Biol.* 37:169-182.
- Nye, J. S., Kopan, R., and Axel, R. (1994). An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Dev.*
25 120:2421-2430.
- Oda, S., Nishimatsu, S., Murakami, K., and Ueno, N. (1995). Molecular cloning and functional analysis of a new activin b subunit: A dorsal mesoderm-inducing activity in *Xenopus*. *Biochem. Biophys. Res. Comm.* 210, 581-588.
- 30 Oliver et al. (1988). *Cell* 55: 1017-24.
- Özkaynak et al. (1990). *EMBO J.* 9:2085-93.

- Padgett et al. (1987). *Nature* 325:81-84.
- Pelton et al. (1990). *Development* 110:609-20.
- Pelton et al. (1989). *Development* 106:759-67.
- Rosa et al. (1988). *Science* 239:783-85.
- 5 Rossant, J., and McBurney, M. W. (1982). The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. *J. Embryol. exp. Morph.* 70:99-112.
- Rudnicki, M. A., and McBurney, M. W. (1987). Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. *In*
- 10 "Teratocarcinomas and Embryonic Stem Cells, a Practical Approach", pp. 19-50. IRL Press, Oxford.
- Ruiz I Altab and Melton (1989). *Nature* 341:33-38.
- Sasai, Y., Lu, B., Steinbeisser, H., and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and BMP-4 antagonistic patterning signals in
- 15 *Xenopus*. *Nature* 376:333-336.
- Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K., and De Robertis, E. M. (1994). *Xenopus* chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 79, 779-790.
- Sato, S. M., and Sargent, T. D. (1989). Development of neural inducing capacity
- 20 in dissociated *Xenopus* Embryos. *Dev. Biol.* 134:263-266.
- Schmidt, J. W., Brugge, J. S., and Nelson, W. J. (1992). pp60src tyrosine kinase modulates P19 embryonal carcinoma cell fate by inhibiting neuronal but not epithelial differentiation. *J. Cell Biol.* 116:1019-1033.
- Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D., and Fischer, W. H. (1990). Activin is a nerve cell survival molecule. *Nature* 344:
- 25 868-870.
- Schulte-Merker, S., Smith, J. C., and Dale, L. (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activity of BVg1 and activin: does activin play a role on mesoderm induction. *Embo* 13:
- 30 3533-3541.

- Smith, J. C. (1993). Mesoderm-inducing factors in early vertebrate development. *EMBO J.* **12**:4463-4470.
- Smith et al. (1990). *Nature* (London) **345**:729-31.
- Spemann, H. (1938). "Embryonic Development and Induction." Yale University
5 Press, New York.
- Spemann, H., and Mangold, H. (1924). Uber Induktion von Embryonanlagen
durch Implantation artfremder Organisatoren. *Arch. mikr. Anat. EntwMech.*
100:599-638.
- Suzuki, A., Theis, R. S., Yamaji, N., Song, J. J., Wozney, J., Murakami, K.,
10 and Ueno, N. (1994). A truncated BMP receptor affects dorsal-ventral
patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **91**:
10255-1259.
- v. d. Eijnden-Van Raaij et al. (1990). *Nature* **345**:732-34.
- van den Eijnden-van Raaij, A. J. M., Feijen, A., Lawson, K. A., and Mummery,
15 C. L. (1992). Differential expression of inhibin subunits and follistatin, but
not of activin receptor type II, during early murine embryonic
development. *Dev. Biol.* **154**:356-365.
- van den Eijnden-van Raaij, A. J. M., van Achterberg, T. A. E., van der
Kruijsen, C. M. M., Piersma, A. H., Huylebroeck, D., de Laat, S. W., and
20 Mummery, C. L. (1991). Differentiation of aggregated murine P19
embryonal carcinoma cells is induced by a novel visceral endoderm-specific
FGF-like factor and inhibited by activin A. *Mech. Dev.* **33**, 157-166.
- Vassalli, A., Matzuk, M. M., Gardner, H. A. R., Lee, K.-F., and Jaenisch, R.
(1994). Activin/inhibin bB subunit gene disruption leads to defects in eyelid
25 development and female reproduction. *Genes Dev.* **8**:414-427.
- Vidricaire, G., Jardine, K., and McBurney, M. W. (1994). Expression of the
Brachyury gene during mesoderm development in differentiation embryonal
carcinoma cell cultures. *Dev.* **120**:115-122.
- Waddington, C. H., and Schmidt, C. A. (1933). Induction by heteroplastic grafts
30 of the primitive streak in birds. *Roux's Arch. EntwMech. Org.* **128**:
522-563.

- Weeks and Melton (1988). *Cell* 51:861-67.
- Whitman and Melton (1989). *Annu. Rev. Cell Biol.* 5:93-117.
- Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L.
(1979). DNA-mediated transfer of the adenine phosphoribosyltransferase
5 locus into mammalian cells. *Proc. Natl. Acad. Sci. USA* 76:1373-1376.
- Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and
inhibition of neural fate by BMP-4. *Nature* 376:331-333.
- Winnier, G., Blessing, M., Labosky, P. A., and Hogan, B. L. (1995). Bone
morphogenetic protein-4 is required for mesoderm formation and patterning
10 in the mouse. *Genes Dev.* 9:2105-16.
- Wozney (1993) "Bone Morphogenetic Proteins and Their Gene Expression," in
Cellular and Molecular Biology of Bone, M. Noda, ed., Academic Press,
Inc., New York.
- Wozney et al. (1988). *Science* 242:1528-34.
- 15 Xu, R. H., Kim, J., Taira, M., Zhan, S., Sredni, D., and Kung, H. F. (1995). A
dominant negative bone morphogenetic protein 4 receptor causes
neuralization in *Xenopus* ectoderm. *Biochem. Biophys. Res. Commun.* 212:
212-9.
- Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M.,
20 Smith, J. C., Heldin, C. H., and Miyazono, K. (1995). Osteogenic
protein-1 binds to activin type II receptors and induces certain activin-like
effects. *J. Cell Biol.* 130, 217-26.

While the invention has been described and illustrated herein by references to
25 various specific material, procedures and examples, it is understood that the
invention is not restricted to the particular material combinations of material, and
procedures selected for that purpose. Numerous variations of such details can be
implied as will be appreciated by those skilled in the art.

WHAT IS CLAIMED IS:

1 1. A method of preventing and/or treating cellular debilitations, derangements
2 and/or dysfunctions and/or other disease states in mammals, comprising
3 administering to a mammal a therapeutically effective amount of a material
4 selected from the group consisting of a gene product of a member of a TGF- β
5 gene family or active fragment thereof, an agent capable of promoting the
6 production and/or activity of said gene product of a member of the TGF- β gene
7 family, an agent capable of mimicking the activity of said gene product of a
8 member of the TGF- β gene family, an agent capable of inhibiting the production
9 of said gene product of a member of the TGF- β gene family, and mixtures
10 thereof, or a specific binding partner thereto, wherein said gene product of a
11 member of the TGF- β gene family induces formation of epidermis.

1 2. The method of Claim 1, wherein the gene product is a bone morphogenesis
2 protein.

1 3. The method of Claim 2, wherein the bone morphogenesis protein is BMP-

1 4. The method of Claim 1, wherein said cellular debilitations result from the
2 infliction of wounds.

1 5. The method of Claim 4, wherein the wounds are a result of surgery.

1 6. The method of Claim 1, wherein the cellular debilitations result from a
2 burn.

1 7. The method of Claim 1, wherein the disease state is the formation of
2 tumors from neural tissue.

- 1 8. A method for producing artificial skin, comprising adding an amount of a
2 gene product of a member of a TGF- β gene family or active fragment thereof
3 sufficient to form epidermis to a culture of cells which is induced to form
4 epidermis.
- 1 9. The method of Claim 8, wherein the gene product is a bone morphogenesis
2 protein.
- 1 10. The method of Claim 9, wherein the gene product is BMP-4.
- 1 11. The method of Claim 9, wherein the amount of BMP-4 is at least 5 ng/ml.
- 1 12. The method of Claim 11, wherein the artificial skin is used for the testing
2 of cosmetics or other externally applied medicaments.
- 1 13. A method of inhibiting neural induction, comprising administering to a
2 mammal an amount sufficient to inhibit neural induction of a material selected
3 from the group consisting of a gene product of a member of a TGF- β gene family
4 or active fragment thereof, an agent capable of promoting the production and/or
5 activity of said gene product of a member of the TGF- β gene family, an agent
6 capable of mimicking the activity of said gene product of a member of the TGF- β
7 gene family, an agent capable of inhibiting the production of said gene product of
8 a member of the TGF- β gene family, and mixtures thereof, or a specific binding
9 partner thereto.
- 1 14. The method of Claim 13, wherein the gene product is a bone
2 morphogenesis protein.
- 1 15. The method of Claim 14, wherein the gene product is BMP-4.

1 16. A method of inducing epidermis formation, comprising administering to a
2 mammal an amount sufficient to induce epidermis formation of a material selected
3 from the group consisting of a gene product of a member of a TGF- β gene family
4 or active fragment thereof, an agent capable of promoting the production and/or
5 activity of said gene product of a member of the TGF- β gene family, an agent
6 capable of mimicking the activity of said gene product of a member of the TGF- β
7 gene family, an agent capable of inhibiting the production of said gene product of
8 a member of the TGF- β gene family, and mixtures thereof, or a specific binding
9 partner thereto.

1 17. The method of Claim 16, wherein the gene product is a bone
2 morphogenesis protein.

1 18. The method of Claim 17, wherein the gene product is BMP-4.

1 19. A method for identifying a compound which is an antagonist of a gene
2 product of a member of a TGF- β gene family for inducing epidermis comprising:
3 A. contacting a biological sample containing cells to be induced with
4 the gene product of a member of a TGF- β gene family or active fragment
5 thereof and a candidate compound; and
6 B. detecting whether the level of epidermis induction by the gene
7 product of a member of a TGF- β gene family is decreased relative to the
8 level of epidermis induction in a control biological sample;
9 wherein a decrease in the level of epidermis induction by the gene product of a
10 member of a TGF- β gene family indicates that the candidate is an antagonist of the
11 epidermis inducing activity of the gene product of a member of a
12 TGF- β gene family.

1 20. The method of Claim 19, wherein the gene product is a bone
2 morphogenesis protein.

- 1 21. The method of Claim 20, wherein the gene product is BMP-4.
- 1 22. The method according to Claim 19, wherein the antagonist is selected from
2 the group consisting of a peptide and an antibody.
- 1 23. The method according to Claim 22, wherein the antagonist is an antibody
2 selected from the group consisting of a polyclonal antibody, a monoclonal
3 antibody, a single chain antibody, an F(ab')₂ fragment of an immunoglobulin, an
4 F(ab') fragment of an immunoglobulin, an Fv fragment of an immunoglobulin,
5 and an Fab fragment of an immunoglobulin.
- 1 24. The method of Claim 19, wherein the antagonist is a dominant-negative
2 activin receptor.
- 1 25. The method of Claim 24, wherein the antagonist is $\Delta 1XAR1$.
- 1 26. A method for inducing expression of an epidermal cell surface marker in
2 epidermal progenitor cells, comprising contacting said cells with an amount of a
3 gene product of a TGF- β gene family or active fragment thereof sufficient to
4 induce expression of said cell surface marker.
- 1 27. The method of Claim 26, wherein the gene product is a bone
2 morphogenesis protein.
- 1 28. The method of Claim 27, wherein the gene product is BMP-4.
- 1 29. The method of Claim 26, wherein said epidermal cell surface marker is
2 Xbra.
- 1 30. A method for inhibiting the expression of an epidermal cell marker in a
2 culture of progenitor cells comprising dispersing said culture such that cell to cell

3 contact is removed for a period sufficient to inhibit expression of said epidermal
4 cell marker.

1 31. The method of Claim 30, wherein the epidermal cell marker is XK81.

1 32. The method of Claim 30, wherein the amount of time is greater than 2
2 hours.

1 33. A method for inducing the expression of a neural cell marker in a culture
2 of progenitor cells comprising dispersing said culture such that cell to cell contact
3 is removed for a period sufficient to induce said neural cell marker.

1 34. The method of Claim 33, wherein the neural cell marker is NCAM.

1 35. The method of Claim 33, wherein the amount of time is greater than 2
2 hours.

1 / 13

FIG.1A

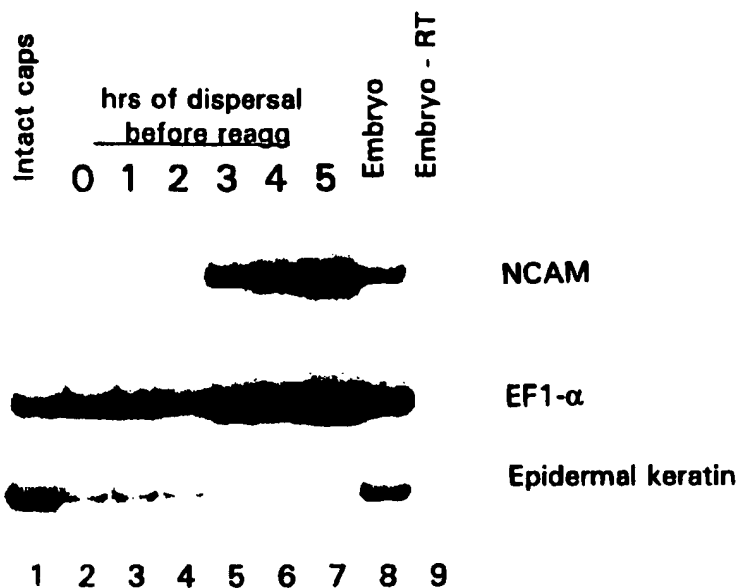
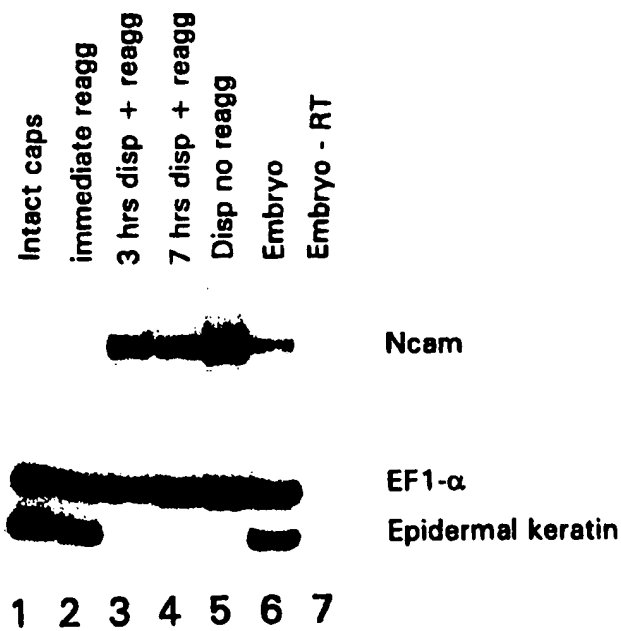


FIG.1B



2 / 13

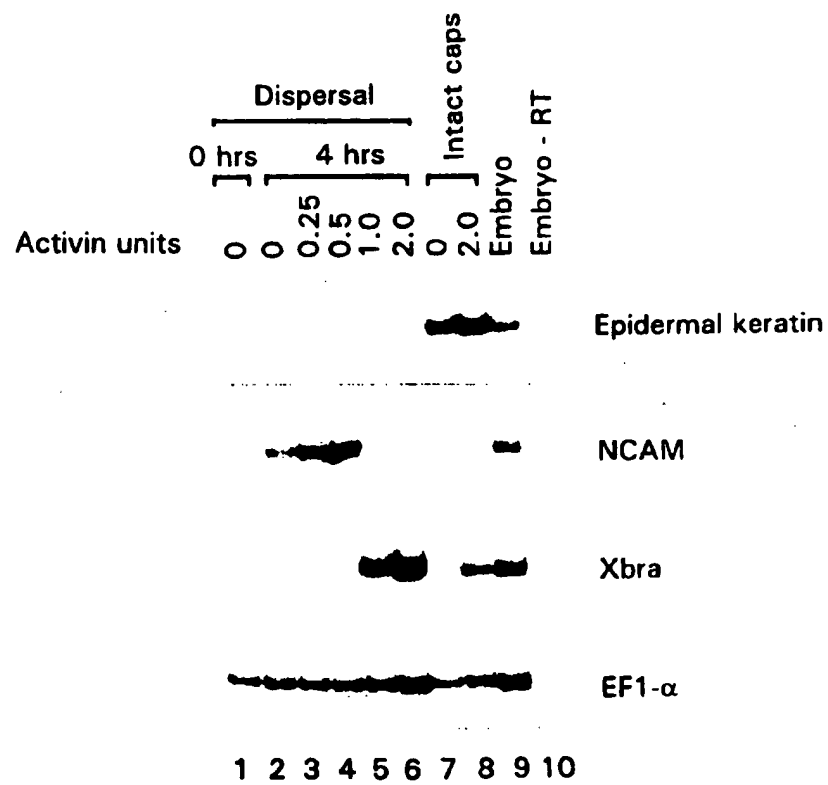


FIG.2

3 / 13

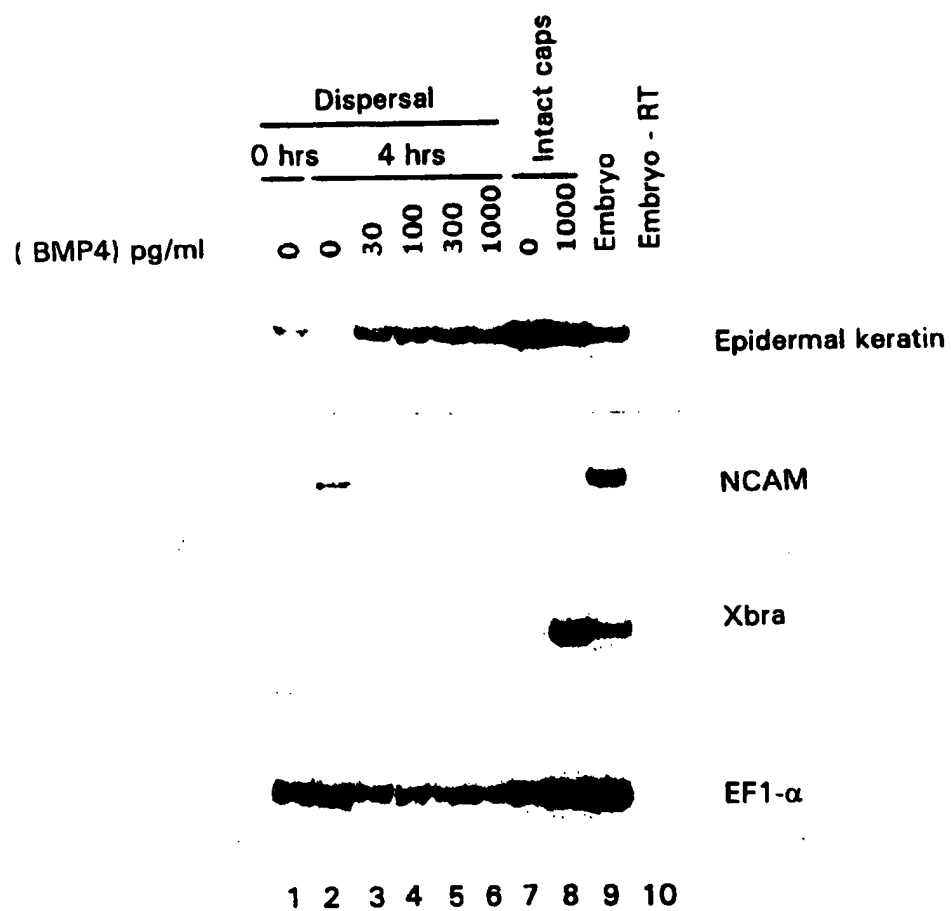


FIG.3

SUBSTITUTE SHEET (RULE 26)

4 / 13

FIG.4A

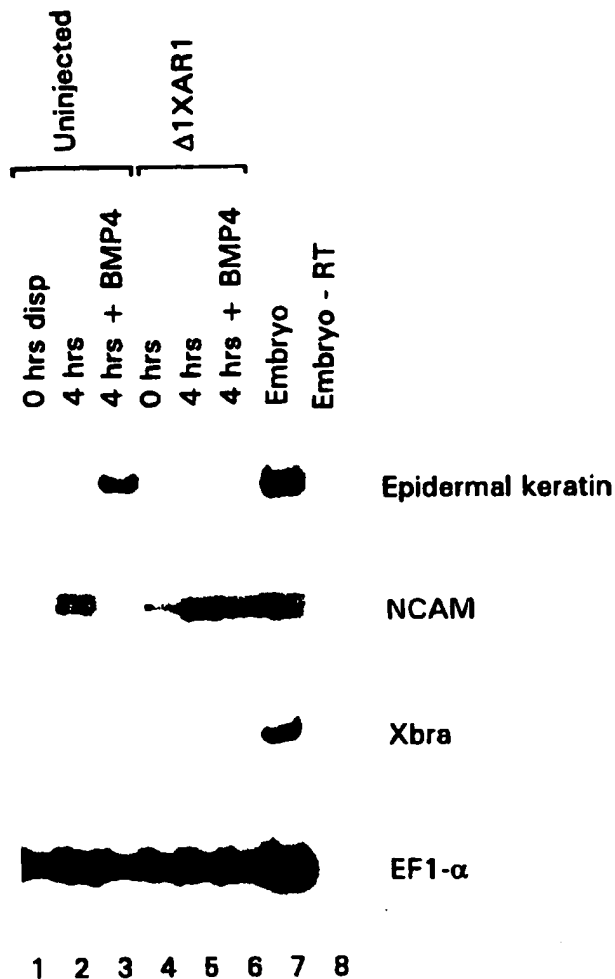
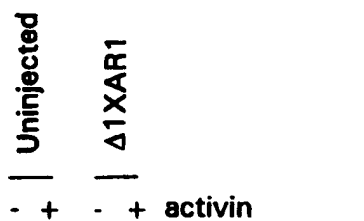
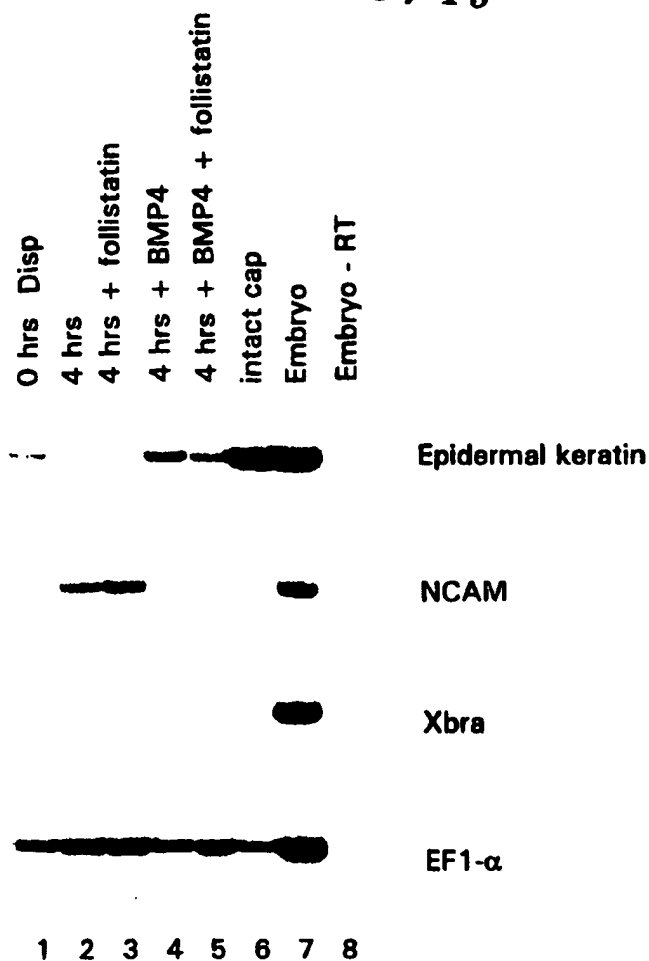


FIG.4B



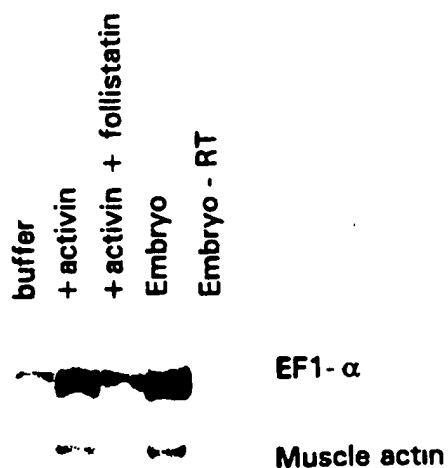
5 / 13

FIG.5A



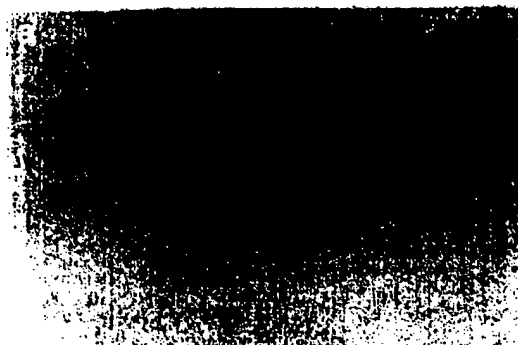
Animal caps

FIG.5B



6 / 13

NF160



pcDNA3

FIG.6A

Light

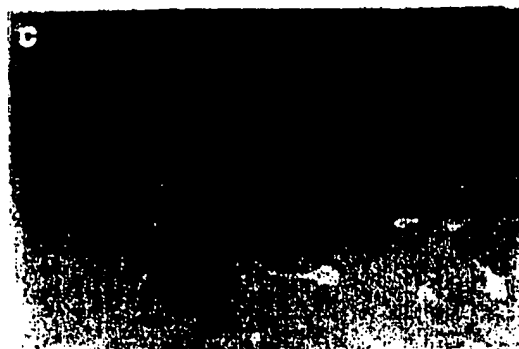


pcDNA3

FIG.6B

7 / 13

NF160



Δ 1XAR1myc

FIG. 6C

Light



Δ 1XAR1myc

FIG. 6D

8 / 13



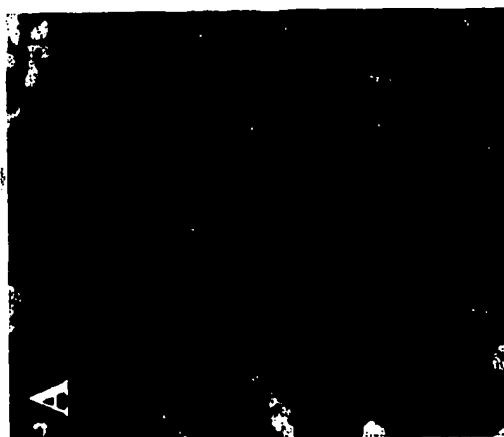
NF 200 + myc

FIG.7C



myc Ab

FIG.7B

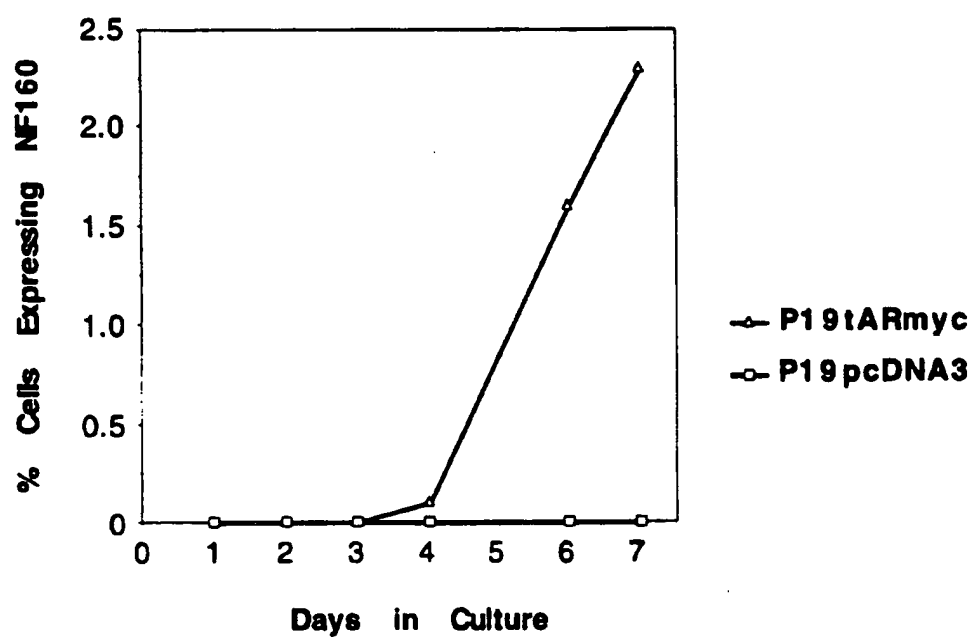


NF 200

FIG.7A

9 / 13

FIG.8



SUBSTITUTE SHEET (RULE 26)

10 / 13

FIG.9A



Control

FIG.9B



Control

FIG.9C



BMP-4
3.6nM

FIG.9D



BMP-4
3.6nM

FIG.9E



RA

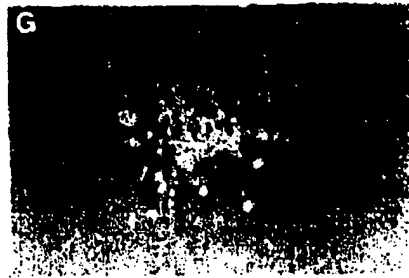
11 / 13

FIG.9F



RA

FIG.9G



RA + BMP-4
0.36nM

FIG.9H



RA + BMP-4
0.36nM

FIG.9I



RA + BMP-4
3.6nM

FIG.9J



RA + BMP-4
3.6nM

12 / 13



FIG.10A



FIG.10B



FIG.10C

D

FIG.10D



FIG.10E

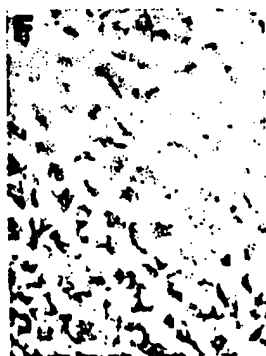


FIG.10F



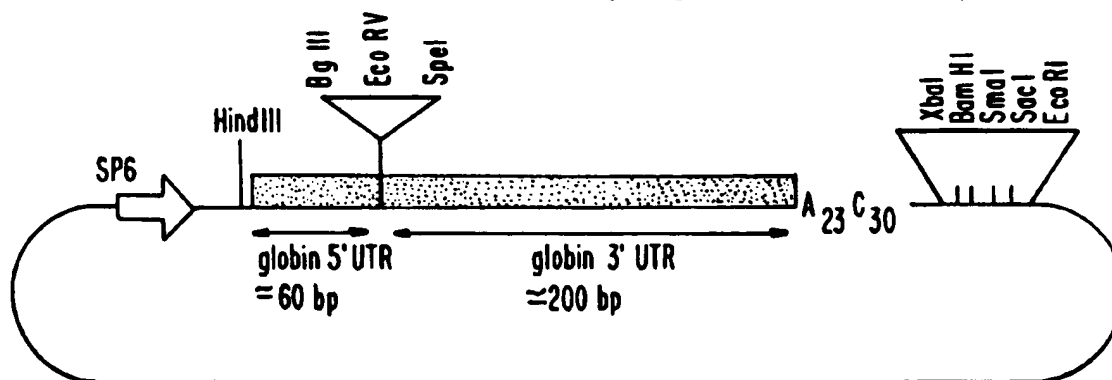
FIG.10G



FIG.10H

13 / 13

FIG. 11A



Sequence of 5' UTR and p.l.

AAGCT TGCTTGTCTTTTTCGAGAAGCTCAGAATAAACGCTCAACTTTGCCAGATCTGATACACTAGT
 Hind III BglII EcoRV SpeI

GACTGACTAGGATCTGGTTACCACTAAACCAGCCTCAAGAACACCCGAATGGAGTCTCTAAGCTACATA
 Stops in 3 frames

ATACCAACTTACACTTTACAAAATGTTGTCCCCCAAATGTAGCCATTCGTATCTGCTCCTAATAAAAAG

AAAGTTTCTTCACATTCTA(23)C(30 TGCAGGTCGACTCTAGAGGATCCCGGGCGAGCTCGAATTCC
 XbaI Bam HI SmaI SacI EcoRI

FIG. 11B

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Publication No
PCT/US 96/04326A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 A61L27/00 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 03200 (CREATIVE BIOMOLECULES) 17 February 1994 see the whole document ---	1,2,13, 14
X	WO,A,95 05846 (GENETICS INSTITUTE) 2 March 1995 see the whole document ---	1-7, 13-15
A	WO,A,92 09697 (CELTRIX LABORATORIES) 11 June 1992 see the whole document --- -/--	1

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

B document member of the same patent family

Date of the actual completion of the international search

19 August 1996

Date of mailing of the international search report

28.08.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

International Publication No

PCT/US 96/04326

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PROC. NATL. ACAD. SCI. U. S. A. (1994), 91(22), 10243-6, 1994, XP002011029 HARLAND, RICHARD M.: "The transforming growth factor.beta. family and induction of the vertebrate mesoderm: bone morphogenetic proteins are ventral inducers" cited in the application see the whole document ---</p>	1
P,X	<p>NATURE (LONDON) 376 (6538). 1995. 331-333, XP002011030 WILSON P A ET AL: "Induction of epidermis and inhibition of neural fate by Bmp -4." cited in the application see the whole document ---</p>	1-35
P,X	<p>GENES & DEVELOPMENT 9 (17). 1995. 2105-2116, XP000578232 WINNIER G ET AL: "Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse." cited in the application see the whole document ---</p>	1-35
P,X	<p>GENES & DEVELOPMENT 9 (23). 1995. 2923-2935, XP000578233 HAWLEY S H B ET AL: "Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction." cited in the application see the whole document ---</p>	1-35
P,X	<p>DEVELOPMENTAL GENETICS 17 (1). 1995. 78-89, XP000578234 HEMMATI-BRIVANLOU A ET AL: "Ventral Mesodermal Patterning in Xenopus Embryos: Expression Patterns and Activities of BMP -2 and BMP -4." cited in the application see the whole document ---</p>	1-35
P,X	<p>NATURE (LONDON) 376 (6538). 1995. 333-336, XP002011031 SASAI Y ET AL: "Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus." cited in the application see the whole document -----</p>	1-35

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 04326

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US96/04326

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Although claims 1-7 and 13-18, and 26-29 as far as it concerns in vivo methods, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Publication No

PCT/US 96/04326

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9403200	17-02-94	AU-B- 4795193	03-03-94
		AU-B- 4797193	03-03-94
		AU-B- 4995593	03-03-94
		AU-B- 5129293	12-04-94
		AU-B- 5129393	12-04-94
		AU-B- 5162393	12-04-94
		AU-B- 5290893	12-04-94
		AU-B- 5590094	24-05-94
		CA-A- 2141554	17-02-94
		CA-A- 2141555	17-02-94
		CA-A- 2141556	17-02-94
		CA-A- 2147598	11-05-94
		EP-A- 0652953	17-05-95
		EP-A- 0653942	24-05-95
		EP-A- 0661933	12-07-95
		EP-A- 0665739	09-08-95
		EP-A- 0661987	12-07-95
		EP-A- 0680334	08-11-95
		EP-A- 0672064	20-09-95
		JP-T- 7509611	26-10-95
		JP-T- 7509720	26-10-95
		JP-T- 7509721	26-10-95
		JP-T- 8501779	27-02-96
		JP-T- 8501558	20-02-96
		JP-T- 8501315	13-02-96
		JP-T- 8503198	09-04-96
		WO-A- 9403600	17-02-94
		WO-A- 9403075	17-02-94
		WO-A- 9406447	31-03-94
		WO-A- 9406399	31-03-94
		WO-A- 9406449	31-03-94
		WO-A- 9406420	31-03-94
		WO-A- 9410203	11-05-94
WO-A-9505846	02-03-95	AU-B- 7868294	21-03-95
		CA-A- 2169191	02-03-95
		EP-A- 0716610	19-06-96
		FI-A- 960809	22-02-96
		NO-A- 960711	22-02-96

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Publication No

PCT/US 96/04326

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9209697	11-06-92	AU-B- 651421	21-07-94
		AU-B- 9141991	25-06-92
		CA-A- 2071912	31-05-92
		EP-A- 0513334	19-11-92
		US-A- 5393739	28-02-95
